The response of the mature central nervous system to traumatic brain injury

By

Catherine Anne Blizzard, BSc (Hons)

Submitted in fulfilment of the
requirement for the Degree of
Doctor of Philosophy

Menzies Research Institute
University of Tasmania (February, 2011)

COPYRIGHT STATEMENT

STATEMENT OF AUTHORITY OF ACCESS

This thesis may be made available for loan and limited copying in accordance with the *Copyright Act 1968*.

Catherine Anne Blizzard

The work submitted in this thesis has been published or submitted for publication as follows;

Blizzard CA, King AE, Vickers JC, Dickson TC. The regenerative ability of mature CNS neurons is intrinsically reduced in NF-L null mice [*manuscript in preparation*]

Blizzard CA, Chuckowree JA, King AE, Hosie KA, McCormack GH, Chapman JA, Vickers JC, Dickson TC (2010). Focal damage to the adult rat neocortex induces wound healing accompanied by axonal sprouting and dendritic structural plasticity. ***Cerebral Cortex*.** [Epub ahead of print].

Murchison EP, Tovar C, Hsu A, Bender HS, Kheradpour P, Rebbeck C, Obendorf D, Conlan C, Bahlo M, **Blizzard CA**, Pyecroft S, Kriess A, Kellis M, Stark A, Harkins TT, Marshall Graves JA, Woods GM, Hannon GJ, Papenfuss AT (2010). The Tasmanian devil transcriptome reveals Schwann cell origins of a clonally transmissible cancer. ***Science*,** 327(5961), 84-87.

Blizzard CA, King AE, Haas MA, Toole DA, Vickers JC, Dickson TC (2009). Axonal shearing in mature cortical neurons induces attempted regeneration and the re-establishment of neurite polarity. ***Brain Research***, 1300:24-36.

Vickers JC, King AE, Woodhouse A, Kirkcaldie MT, Staal JA, McCormack GH, **Blizzard CA**, Musgrove RE, Mitew S, Liu Y, Chuckowree JA, Bibari O, Dickson TC (2009). Axonopathy and cytoskeletal disruption in degenerative diseases of the central nervous system. ***Brain Res Bull***, 80(4-5), 217-23.

King AE, Dickson TC, **Blizzard CA**, Woodhouse A, Foster SS, Chung RS, Vickers JC (2009). Neuron-glia interactions underlie ALS-like axonal cytoskeletal pathology. ***Neurobiol Aging***. [Epub ahead of print].

Blizzard CA, Haas MA, Vickers JC and Dickson TC (2007). Cellular dynamics underlying regeneration of damaged axons differs from initial axon development. ***European Journal of Neuroscience****,* 26, 1100-1108.

King AE, Dickson TC, **Blizzard CA**, Foster SS, Chung RS, West AK, Chuah MI and Vickers JC (2007). Excitotoxicity mediated by non-NMDA receptors causes distal axonopathy in long-term cultured spinal motor neurons**.** ***European Journal of Neuroscience****,* 26, 2151-2159.

SUMMARY

The mature central nervous system (CNS) is unable to repair following traumatic brain injury (TBI). Intervention is difficult as the pathobiology of the brain following injury is a complex sequence of events that needs to be fully elucidated. Understanding the endogenous mechanisms evoked by the damaged brain when attempting repair from injury is vital for devising effective therapies to treat brain injury. The current thesis is based upon the hypotheses that ultimately, recovery following trauma will require the induction of neurogenesis and either appropriate regeneration or compensatory plasticity of pre-existing neural pathways and that the mechanisms underlying regeneration of mature axons is fundamentally different to developmental growth.

This thesis investigated the reactive and regenerative alterations associated with the neural response to physical injury in the adult mammalian brain. This thesis studies are focused upon the potential for regeneration following injury and how comparable regenerating neurons characteristics are to their developmental counterparts. It investigated the alterations within the damaged neurons and the surrounding brain area which may be indicative of an intrinsic capacity for regeneration including frank neuronal replacement following injury, and the role of the neuronal cytoskeleton in neuronal regenerative events, as the mechanisms underlying these processes are currently poorly understood.

This thesis demonstrated that heterogenous populations of cultured cortical neurons are able to survive and regenerate following severe structural injury, suggesting that neurons have an intrinsic capacity for regeneration, regardless of the mode of injury. Additionally, alteration of the intrinsic cytoskeletal environment, through the knockout of the neurofilament light chain protein, decreased the regenerative ability of mature neurons, providing further evidence for the intrinsic regulation of regeneration. Results from this thesis indicate that the growth cones of regenerative sprouts differ from their developmental counterparts in their predominant morphology, their dynamic behaviour and their ability to respond to critical growth factors. These differences between the regenerating and developing growth cones may account for the inability of regenerative

sprouting axons to make accurate pathway decisions and successfully respond to trauma.

The presence of neurogenesis was investigated following structural injury *in vitro,* which indicated no evidence of neurogenesis following injury. While focal brain injury *in vivo* induced proliferation of neural progenitors, immunohistochemistry confirmed that astrocytic but not neuronal, cell proliferation was evoked by focal injury, consistent with the *in vitro* data. However focal injury induced an axonal regenerative response into the injury site, which was neuronal cell type specific, and significant remodelling in a subpopulation of interneurons away from the injury, demonstrating that the adult cortex is capable of significant remodelling following brain injury.

These studies address the central issues examining the potential for neuronal repair and appropriate regeneration with an emphasis on developmental versus regenerative growth. This thesis provides significant insight into why regenerative attempts are limited or aberrant and how responses exhibited by cortical neurons are specific to subpopulations. This knowledge will provide the evidence base for new therapeutic strategies to improve clinical outcomes for sufferers of TBI.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors Dr Tracey Dickson, Dr Anna King and Professor James Vickers for their endless guidance, support, generosity and humour, who without, this thesis would not have been possible.

I would like to thank my colleagues within the NeuroRepair group (Dr Roger Chung, Dr Jyoti Chuckowree, Dr Matilda Haas, Dr Adele Woodhouse, Dr Heather McGee, Dr Jerome Staal, Dr Oliver Bibari, Micheal Pankhurst, Stan Mitew, Samuel Alexander, Katherine Hosie, Ruth Musgrove and Yao Liu) for their support and friendship. I would like to thank my friends and colleagues within Menzies Research Institute for the shared expertise and beverages.

I would like to thank Dr Mala Rao and Dr Stephen Ginsberg from the Nathan Line Institute (USA) and Professor Jean-Pierre Julien from Chul Research Centre (Canada) for providing the transgenic mouse line with a null neurofilament light chain mutation.

Finally I would like to thank my parents, Cheryl and Leigh Blizzard and my partner, Damien Jenkins who have provided me with limitless patience, encouragement and hot meals.

TABLE OF CONTENTS

ABBREVIATIONS

ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
BrdU	5-Bromo-2'-deoxyuridine
CO_2	carbon dioxide
°C	degrees Celsius
CNS	central nervous system
DIC	differential interference contrast
DIV	days *in vitro*
ERM	ezrin-radixin-moesin
g	gram
GABA	gamma-Aminobutyric acid
GAP-43	Growth-associated protein 43
GDNF	glial derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
IgG	immunoglobulin G
IgM	immunoglobulin M
kDa	kilo Dalton
L	Litre
µl	micro litre (10^{-6}l)
µm	micrometre (10^{-6}m)
µM	micro molar (10^{-6}M)
M	Molar
ml	millilitre
mm	millimetre
MAP	microtubule associated protein
MAP-2	microtubule-associated protein 2
NF	neurofilament
NF-H	neurofilament triplet high molecular weight subunit
NF-M	neurofilament triplet medium molecular weight subunit

NF-L	neurofilament triplet light molecular weight subunit
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PFA	paraformaldehyde
PI	post-injury
PNS	peripheral nervous system
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SVZ	subventricular zone
TBI	traumatic brain injury
TBS	tris buffered saline
TIP	microtubule plus end tracking protein
TTBS	tris buffered saline with tween
VZ	ventricular zone

1 INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability in persons under the age of 45 years, in developed nations. (Adams, 1995; Egeler-Peerdeman, 1993; Povlishock, 2005; Maas et al., 2008). TBI can affect individuals at any age, and encompasses any form of acquired injury to the head that causes central nervous system (CNS) damage. Currently in Australia the most common causes of TBI include transport accidents, falls, collisions with objects and water related injuries (The Victorian Neurotrauma Initiative, 2009). The incidence of TBI is rising rapidly on a global scale, for two main reasons (Maas et al., 2008). Firstly there is an increasing use of motor vehicles in low to middle income countries. Secondly, for persons over the age of 65 accidental falls represent the most common form of injury and epidemiological evidence indicates that as the population ages an increase in the number of head injuries from falls is also occurring. TBI equates to a major health and socio-economic burden, as head injury often affects individuals in the most productive years of their lives (Duff, 2001; Finnie and Blumbergs, 2002). The current cost burden of TBI in Australia alone is estimated to be 8.6 billion dollars (The Victorian Neurotrauma Initiative, 2009).

While TBI incidence is rising rapidly on a global scale, there still remains no effective treatment. The inability to recover following TBI can be accounted for by the relatively limited capacity of the mature CNS to repair and regenerate. This is in distinct contrast to both the peripheral nervous system (PNS) and the developing CNS, which both demonstrate remarkable regenerative capacities. Hence the basis for the inability of the mature CNS to recapitulate these systems remains a pertinent research question at the forefront of neuroscience research.

Moderate to severe head injury often causes overt brain damage resulting in widespread neuronal dysfunction. However, even mild brain trauma can elicit a cascade of cellular changes that culminates in neuropathology. Additionally, head injury has been implicated in the development of neurodegenerative diseases, such as Alzheimer's disease, later in life (Smith et al., 1999; Graham et al., 2000; King et al., 2000a; Vickers

et al., 2000; Jellinger et al., 2001). However, there are varying degrees of focal and diffuse injury that can occur in TBI, affecting various regions of the brain (reviewed in Farkas and Povlishock, 2007), making the pathobiology complex and intervention difficult. Due to the array of mechanisms evoked by TBI and individual patient differences, the response of the adult brain remains to be fully elucidated. The sequence of events following injury must be fully comprehended before successful therapeutic intervention can occur. Understanding the structural and functional architecture of the mammalian brain is key in endeavouring to comprehend the pathobiology of TBI.

1.1 THE MAMMALIAN BRAIN

The mammalian nervous system is a network of nerve cells and fibres that infiltrates virtually all areas of the body. The nervous system is broadly divided into the CNS and PNS. The brain, together with the spinal cord makes up the CNS and can be is divided into grey and white matter. Grey matter consists predominantly of neuronal cell bodies and dendrites and is highly vascularised, whereas white matter is composed primarily of axons, the majority of which are ensheathed by specialised myelinating glial cells. The brain can further be subdivided into the cerebrum, cerebellum and brainstem. The cerebrum is comprised of a pair of cerebral hemispheres, their surface thinly coated by the cerebral neocortex. The cerebral neocortex, which is between just two and four millimetres thick, is made up of an estimated 25 billion neurons, and is responsible for up to 80% of the function of the human brain (Markram et al., 2004; Nolte, 2009).

1.1.1 Neurons of the neocortex

During evolution, higher vertebrates developed a large cerebral cortex, with the most recent development being the outer neocortex (meaning new cortex) (Medina and Abellan, 2009; Rakic, 2009). The neurons within the neocortex can be generally categorised into two groups, pyramidal cells and cortical interneurons, or non-pyramidal cells, based primarily on morphology (Hendry, 1989; Molnar et al., 2006). Interactions between cortical pyramidal cells and interneurons facilitate highly integrated cortical processing. A notable feature of the neocortex is the sorting of neurons in the outer region into six horizontal layers, denoted layer I through VI, from the most superficial to deep layer (Silberberg et al., 2005; Nolte, 2009) (Figure 1.1). Laminae are

determined by variations in cell body size and density throughout the cortical depth (Parnavelas, 2000) (Figure 1.1). Each layer can be distinguished based on the predominant neuronal cell population. Layer I, the molecular layer, is relatively cell free. Layers II and IV, the external and internal granular layers are comprised of relatively small locally projecting interneurons, whereas layers III and V contain characteristic large cortical pyramidal cells and are thus designated the external and internal pyramidal layers, respectively. In addition to the laminar organisation, the neocortex also has a distinct columnar organisation with several structures, including the apical dendrites of pyramidal cells, cortical afferents, axons of some intra-cortical cells and the cell bodies of cortical neurons, arranged horizontally and perpendicular to the cortical surface.

Synaptic transmission within the neocortex follows a distinct and highly complex pattern, with integration both within and between the cortical laminae (reviewed by Silberberg et al., 2005). Briefly, thalamic input primarily enters layer IV and is passed onto layer III pyramidal cells that, in turn, project to layer II. Layers II and III also receive input from association brain regions and provide descending input to layer V pyramidal cells. Depending on their type, layer V pyramidal neurons either project to subcortical regions, comprising the major source of neocortical output, or to the contralateral hemisphere. Layer V pyramidal cells also project to layer VI, forming both cortico-cortical interconnections and corticothalamic feedback circuits. The majority of neocortical pyramidal cells also project their dendrites into layer I, forming an additional level of cortical processing and integration.

1.1.1.1 Pyramidal neurons

Pyramidal neurons are the primary glutamatergic excitatory cells of the neocortex (Elston et al., 2001). Pyramidal neurons have large cell bodies ranging from 10-50 micron in diameter (Lynch, 2006), extensive dendritic arborisations that have specialised synaptic protrusions termed dendritic spines and one long projecting axon. The pyramidal-shaped soma elaborates a prominent apical dendrite that extends up towards the pial surface. Basal dendrites extend horizontally from the base of the soma. The axon of pyramidal cells, which is usually myelinated, generally extends from the

base of the soma, opposite to the apical dendrite, but may instead arise from a basal dendrite (Feldman, 1984). Pyramidal neurons are primarily contained within layers II to VI and comprise approximately 70-80% of all neocortical neurons (Peters and Jones, 1984; Markram et al., 2004; Voelker et al., 2004; Silberberg et al., 2005). The pyramidal cell layers contain predominantly neurons that project long axons to other regions of the CNS.

1.1.1.2 Interneurons

Interneurons incorporate a sub-population of cortical neurons with a vast array of morphological features and molecular identities (Parnavelas, 2000). Knowledge on the various subtypes of gamma -aminobutyric acid-ergic (GABAergic) interneurons have been predominately derived from immunohistochemical studies looking at the localisation of the three calcium binding proteins (Xu et al., 2010); calbindin (Hof and Morrison, 1991; Seress et al., 1993; Leuba et al., 1998), calretinin (Hof et al., 1993; Fonseca and Soriano, 1995; del Rio and DeFelipe, 1997) and parvalbumin (Aria et al., 1987; Hof et al., 1991; Hof and Nimchinsky, 1992; Hof et al., 1999). The three types of calcium binding proteins are found within mutually exclusive populations of interneurons. Calbindin positive proteins are found mainly in granule cells of the dentate gyrus (Seress et al., 1993), interneurons in cortical layers II/III, and to a lesser extent in layers V/VI (Leuba et al., 1998). The interneuron population positive for parvalbumin is found in cortical layers II and III and the molecular layer of the dentate gyrus and throughout the subiculum (Seress et al., 1993; Leuba et al., 1998). Calretinin positive interneurons reside predominately in the superficial portions of cortical layers II and IV (Leuba, 1998). Other subpopulations of neurons immunopositive for calcium binding proteins occur in subcortical regions as well as the cingulate cortex and amygdala (Hof and Nimchinsky, 1992; Hof et al., 1999). Interneurons are predominantly inhibitory (mostly GABA inhibitory) in function and play a vital role in modulating cortical output (Segal and Barker, 1984; Markram et al., 2004; Otsuka and Kawaguchi, 2009; Xu and Callaway, 2009). Interneurons' axons essentially do not leave the immediate vicinity of the soma, and therefore modulate the intrinsic activity of the directly surrounding pyramidal cells (Kawaguchi and Kubota, 1997; Otsuka and Kawaguchi, 2009; Xu and Callaway, 2009). These neurons are morphologically

identified by the absence of a prominent apical dendrite and the presence of few, if any, dendritic spines; although specific sub-types of interneurons may have dendritic spines equivalent to pyramidal neurons (Fairen et al., 1984; Markram et al., 2004). Thus, interneurons have less extensive dendritic arborisations, a short, locally projecting axon and a small cell body (Hof et al., 1999; Markram et al., 2004; Nolte, 2009).

1.2 DEVELOPMENT OF THE MAMMALIAN BRAIN

The complexity and relative large size of the human brain elicits the mental capabilities that distinguish humans from all other species. During development a relatively simple neural tube is transformed into this intrinsically complex organ through a stringently controlled pattern of neuronal proliferation, migration and maturation (Noctor et al., 2004; Corbin et al., 2008). The process of development of the cortex, corticogenesis, has been widely described (see reviews by Frisén et al., 1998; Parnavelas, 2000; Nadarajah and Parnavelas, 2002; Honda et al., 2003; Kubo and Nakajima, 2003; Marshall et al., 2003). Neurons destined to form the neocortex are generated at the ventricular lining and periventricular zones. More specifically, cortical pyramidal neurons are generated in the germinal ventricular zones of the dorsal telencephalon, whereas sources of cortical interneurons include the lateral ganglionic eminence and medial ganglionic eminence of the ventral telencephalon (Parnevelas, 2000; Kreigstein and Noctor, 2004; Wonder and Anderson, 2006). The neocortex develops in two distinct stages (Figure 1.2). Firstly post-mitotic neurons migrate toward the margin of the cerebral wall to form the primordial plexiform layer, or preplate. New born neurons migrate into the preplate and as they accumulate, form a new series of layers called the cortical plate, which thereby splits the preplate into the superficial marginal zone (layer I) and a deeper layer, the subplate (layers II-VI); the second stage of development. The later born post-mitotic neurons that form layers II-VI of the cortical plate are arranged in an ‘inside-out’ sequence, where new cells migrate through the existing cells before stopping at the top of the plate (reviewed by Parnevalas, 2000; Kreigstein and Noctor, 2004). The subplate and ventricular zone are separated by the intermediate zone, which will eventually contain the axons of the cortex (Bielas and Gleeson, 2003).

There are two main modes by which new neurons migrate, known as radial and tangential migration (Naradajah et al., 2001). Neurons of the cerebral cortex, specifically those destined to become pyramidal neurons, take on a mode of radial migration, moving in a direction perpendicular the pial surface, guided by radial glia (Huang, 2009). Interneuronal precursors of the cerebral cortex, on the other hand, undergo tangential migration, moving from the ganglionic eminence in a direction parallel to the pial surface (Huang, 2009). However not all precursor neurons are able to reach their appropriate destination by radial migration and tangential migration alone. A third mode of migration, termed 'multipolar migration' also exists (Tabata and Nakajima, 2003) characterised by net radial migration, interspersed with episodes of tangential migration. After completing migration a neuron enters a phase of growth, the first stage of which involves developing multiple neurites (Dotti et al., 1988; Deitch and Banker, 1993). Following initiation, one neurite will polarise and grow more rapidly than the other neurites to form the axon (Dottie et al., 1988) while the other shorter, highly branched processes become dendrites. Axons then extend through a carefully selected pathway to find their final, specific target where they form synapses. The highly precise neuronal network of the brain is established through temporal and spatial regulation of guidance molecules that direct growth of the axons along specific pathways (Dickson et al., 2002; Osterfield et al., 2003).

1.2.1 Neuron outgrowth and the cytoskeleton

The extensive neuronal network of the brain is developed by the highly accurate pathway selection of axons. Growth cones are transient structure present on the tips of elongating axons that enable this stringent pathway selection (Figure 1.3). The growth cone is present for a finite time during development, between the commencement of neuronal differentiation and the phase of synaptogenesis. Fundamentally, growth cones translate extracellular guidance cues into a mode of either extension or retraction, depending on whether the cue is attractive or repulsive. Upon reaching the desired target the growth cone immobilises, remodels and forms a synapse.

Growth cones have both sensory and motor capabilities, which enables the integration of environmental cues to be translated into path finding behaviour, such as outgrowth,

retraction, stalling, turning and fasciculation (for review see Tessier-Lavigne and Goodman, 1996; McFarlane, 2000; Suter and Forscher, 2000; Gordon-Weeks, 2004) (Figure 1.3). Growth cones undergo dramatic shape changes ranging from large fan like shapes to a more stream like structure (Kalil, 1996). Morphological analysis of growth cones in different regions of developing central and peripheral pathways in a variety of species has demonstrated that the change in morphology is related to the environmental cues (Halloran and Kalil, 1994; Kalil, 1996; Skaliora et al., 2000). When growth cones track along straight trajectories, such as the internal capsule, they exhibit simple streamline forms (Skaliora et al., 2000). The internal capsule represents a compact curved sheath of cortical projection axons and as such the axons grow fast in direct trajectories with minimal pausing and exploring of their surrounding. When encountering a decision region, such as the ventral intermediate zone for the cortical projection axons, growth cones undergo dynamic alterations in morphology becoming large and considerably more complex in shape. When reaching a decision region such as the ventral intermediate zone, growth cone motility slows down with extensive exploration of the environment and pausing, which can last up to several hours (Skaliora et al., 2000).

Environmental guidance cues are either attractive cues that the growth cone will turn toward, or repulsive guidance cues that cause a collapse in the growth cone. It is important to note however, that growth cone turning, and advances, are actually regulated independently, except when the guidance cue is particularly strong (Sanford et al., 2008). Guidance cues in the growth cone environment can be either anchored in the extracellular matrix, on a cell membrane or presented as a concentration gradient that diffuses from an intermediate or final target source (Chilton, 2006). Growth cones are responsible for translating extracellular guidance cues, which dictate trajectory into cytoskeletal rearrangements, which establishes axonal morphology and route taken (Tanaka and Sabry, 1995; Nakamura et al., 2000; Pak et al., 2008; Hung et al., 2010). Guidance molecules such as neurotrophins, semaphorins, ephrins, netrins and slits are in abundance within the developing brain (Dickson, 2002; Sanchez-Camacho and Bovolenta, 2009; Hung et al., 2010; Yukawa et al., 2010). Neurotrophins act as attractants and the semaphorins, ephrins and slits act predominately as a repellent,

however they can also be attractive in some contexts. Netrins can act as either an attractant or repellent, depending on the context (Dickson, 2002). Binding to and activation of receptors for these guidance molecules in the growth cone cytoplasm triggers intracellular signalling pathways directed predominately at the growth cone cytoskeleton (Gallo and Letourneau, 2004; Kalil and Dent, 2005; Zhou and Schnider, 2006). However, it is important to note that the intracellular environment of the growth cone also plays a role in the response to guidance molecules. Specifically cyclic nucleotide levels, the intracellular calcium concentration and membrane potential all moderate the response of the growth cone to a certain guidance cue (Mcfarlane, 2000; Wen et al., 2004).

Growth cones are highly structured due to the diversity and organisation of their cytoskeletal components. Within the axon and the growth cone, the cytoskeleton is classified into three components, the microtubules, microfilaments and neurofilaments (Figure 1.4). The central zone of the growth cone, including the termination of the neurite shaft, consists predominantly of bundles of microtubules (reviewed in Gungabissoon and Bamburg, 2003; Gordon-Weeks, 2004). The central zone is also rich in membranous organelles required for membrane elaboration (Figure 1.3). The peripheral area, specifically the lamellipodia and filopodia, are dominated by microfilaments, in the form of filamentous actin (F-actin) (Figure 1.3). While microtubules reside predominately in the central zone and F-actin in the peripheral zone, there is extensive overlap of these cytoskeletal components between the two regions, within the transitional zone. Within growth cones, microtubules and actin are the cytoskeletal components responsible for movement and are the ultimate targets of directional signalling.

1.2.1.1 Microtubules within the developing axon and growth cone.

Microtubules are long hollow structures comprised of protofilament aggregates of tubulin. Two subunits of tubulin, α and β tubulin, polymerise to form these protofilaments. Thirteen protofilaments then aggregate together to form the microfilament. Within the axon, microtubules participate in the active process of axonal transport. This allows fast transport of macromolecules and organelles to the distal area

of axons and to the growth cone. The role of microtubules in axon transport has been compared to a railroad track, on which two microtubule associated ATPases serve as "motors" (Waterman-Storer, 1997; Pfister, 2000; Hollenbeck and Saxton, 2005). Within the axonal shaft, microtubules form a dense parallel array that upon entering the growth cone splays apart. Studies over the past decade convincingly indicate that dynamic microtubules within the growth cone play a crucial role in growth cone steering (Buck and Zheng, 2002).

Within the growth cone microtubules reside principally in the central domain of the growth cones, however are present at times in both the transitional and peripheral zone. Microtubules within the central domain are frequently in a fan like splayed out orientation, however in slow growing growth cones and growth cones that are pausing, they become bundled and form loops (Sabry et al., 1991; Tanaka and Kirschner, 1991; Dent and Kalil, 2001). The majority of microtubules enter the central domain of the growth cone through the terminal end of the neurite shaft. The microtubules contained within the central zone are in a state of dynamic instability, which means they are constantly undergoing rapid periods of growth and shrinkage (Buck and Zheng, 2002, Gordon-Weeks, 2004; Chilton, 2006; Lee and Suter, 2008; Lowery and Vactor, 2009). The dynamic growth of microtubules that results from this dynamic instability allows the microtubules to respond to the growth cones' intracellular environment, namely F-actin. The dynamic growth is a result of the process of end polymerisation of heterodimer subunits of tubulin. Tubulin heterodimers are intrinsically polar polymers that associate in a head to tail formation within each protofilament (Gordon-Weeks, 2004). The positively charged (plus) end of the microtubules is orientated to the distal area of the growth cone and the negatively charged (minus) ends are orientated proximally toward the axon shaft. Both the plus and minus end have potential for growth, however the plus ends have a far greater capacity and growth occurs at a more rapid rate than the minus ends. Orientation of the fast growing plus ends of the microtubules toward the distal part of the growth cone allows for the extension of the growth cone along a trajectory. Microtubules are in a constant state of dynamic instability, alternating between slow growth at the minus end and rapid growth at the plus end. The transition from slow growth at the proximally orientated minus end to

rapid growth at the distally orientated plus can result in complete depolymerisation (termed a catastrophe) and retraction of the microtubule, unless rescued to another transition of slow growth. This dynamic instability is likely to be structurally due to a loss of a structural cap (GDP β-tubulin) at the end of the microtubule (Gordon-Weeks, 2004). Microtubules dynamics is regulated by two families of proteins, microtubule associated proteins (MAPs) and plus end tracking proteins (TIPs). MAPS regulate dynamic instability by increasing the rate of rescue and decreasing the rate of catastrophes (Pryer et al., 1992). TIPs are located on the plus end of growing microtubules and are involved in facilitating the microtubule and F-actin interaction (Geraldo et al., 2008). Tyrosination of microtubules is also required for successful microtubule and F-actin interactions, and the knock out of ligases responsible for tyrosination leads to a decrease in ability to path find (Marcos et al., 2009).

The formation of microtubules and promotion of the bundle formation (stabilisation) and conversely the dissolution of the bundles and aggregate formation (destabilisation) of microtubules has also been shown to be important in the turning of growth cones. Local stabilisation and destabilisation of microtubules via the drugs taxol and nocodazol respectively has been shown to alter growth *in vitro* (Buck and Zheng, 2002; Chuckowree and Vickers, 2003). When microtubules are locally stabilised the growth cone turns toward the site of stabilisation and reciprocally local destabilisation results in the turning away from the site of destabilisation. The actin cytoskeleton actively participates in microtubule initiated growth cone turning (Buck and Zheng, 2002) with local microtubule stabilisation and growth within the growth cone being dependent upon the F-actin interaction. Microtubules growing toward the front of the growth cone transverse the transitional zone and get into the peripheral domain. This allows interaction between the microtubules and F-actin within the peripheral zone, microtubules preferentially interacting with the filopodial F-actin bundles (Lee and Suter, 2008; Geraldo et al., 2008; Geraldo and Gordon-Weeks, 2009; Lowery and Vactor, 2009).

1.2.1.2 Microfilaments within the developing axon and growth cone

The second principal cytoskeletal component within developing axons and growth cones is the microfilaments, specifically F-actin. Monomeric globular, G-actin subunits polymerise to form filamentous, F-actin. Morphology of the peripheral domain is largely determined by the F-actin and its organisation into filopodia and lamellipodia. Filopodia are narrow, cylindrical and highly motile extensions of the growth cone. They interact with the environment, having the capacity to extend tens of microns from the growth cone. F-actin forms rod-like parallel bundles within the filopodium. Lamellipodia, the veil like membranous extensions situated between the filopodia, contain a meshwork of connected and branched F-actin. The predominance of lamellipodia or filopodia correlates with the translocation rate of the growth cone (Argiro et al., 1984; reviewed in Pak et al., 2008). These dynamic structures are constantly extending and retracting, enabling the growth cone to explore the extracellular environment for potential guidance cues.

The guidance and growth of growth cones is determined by the interactions of filopodia and the F-actin, through transmembrane receptors, with the extrinsic environment. The F-actin present within in the filopodia also undergoes dynamic assembly, which is the precursor for membrane protrusion and extension (Forscher and Smith, 1988). Polymerised F-actin within the peripheral domain is under constant retrograde transport, powered by myosin motors, towards the central domain of the growth cone (for reviews see Suter and Forscher, 2000; Gallo and Letourneau, 2004). During this retrograde transport, F-actin is dynamic, undergoing filamentous turnover; near approach of the central domain, filaments undergo depolymerisation and actin subunits are recycled for future polymerisation to F-actin. The surface of the filopodium is rich in transmembrane proteins which act as receptors for cell adhesion molecules. The adhesion of the filopodia to a suitable substrate activates transmembrane signalling causing F-actin polymerisation, and results in elongation of the filopodium. Outward movement of an axon is inversely proportional to retrograde F-actin flow (Hely and Willshaw, 1998). The slower the retrograde flow of F-actin toward the central zone of the growth cone, the faster the microtubule invasion into the target area.

Axonal outgrowth is achieved by an interaction between the actin and microtubules cytoskeleton, which creates stretch of the axon (Lamoureux et al., 2010). A growth cone in a straight trajectory contains actin bundles uniformly distributed throughout the lamellipodia, however a concentration of the actin meshwork occurs at the leading edge. Assembly of F-actin occurs at this leading edge and at the tips of the filopodium. The retrograde F-actin flow from this area is at a diminished rate compared to the polymerisation rate of the microtubules within the central domain. This allows the growing microtubules to extend into the peripheral domain where there is decreased retrograde F-actin flow (Buck and Zheng, 2002). Contact with an actin bundle within the peripheral domain induces the microtubule to advance in a linear path along the bundles toward the leading edge; microtubules that do not come in contact with actin are swept back toward the central domain (for review see Zhou and Cohan, 2004). The microtubule and F-actin interaction results in outgrowth of the growth cone in the direction of the leading edge. The majority of known guidance molecules affect direction of growth through the rapid reorganisation of the actin/microtubule cytoskeleton in the peripheral zone (Cohen-Corey and Fraser, 1995; Gallo and Letourneau; 2003; Hou et al., 2008; Geraldo and Gordon-Weeks, 2009; Hall and Lalli, 2010), further highlighting the importance of the actin/microtubules reorganisations.

1.2.1.3 Intermediate filaments within the developing axon and growth cone

The intermediate filaments are integral to the structural morphology of the neuron and axon. Neurofilaments provide tensile strength as well as stabilising neuronal structure (Hamberger et al., 2003). They are involved in controlling axonal calibre, an important factor in the speed of conductance down the axon. The neuronal intermediate filament protein family is made up of neurofilament light chain (NF-L), middle chain (NF-M) and heavy chain (NF-H), α-internexin and peripherin (Lee et al., 1993; Lee and Cleveland, 1996). Neurofilament triplet proteins, with α-internexin comprise the neuronal intermediate filament family of type IV intermediate filament proteins (Lariviere and Julien, 2004) (Figure 1.5A). During development, two more intermediate filaments vimentin and nestin are also present within CNS neurons (Gates et al., 1995; Arnold and Trojanowski, 1996). These proteins share a common structural organisation comprising a central α helical rod domain flanked by amino terminal head

and carboxy-terminal tail domains (Figure 1.5A). Together NF-L, NF-M and NF-H copolymerise to form neurofilaments. The expression of intermediate filaments varies throughout development and facilitates the transition of a developing neuron, from a highly plastic mitotic cell, to a post-mitotic highly polarised structure with distinct morphological features (Nixon and Sihag, 1991; Nixon and Shea, 1992). Specifically, the first intermediate protein to be expressed is vimentin, in undifferentiated cells (Bignami et al., 1982; Cochard and Pauin, 1984). Later in development as the undifferentiated cells form neuroblasts the expression of nestin, α-internexin and peripherin is initiated (Porttier et al., 1983; Parysek and Goldman, 1987; Kaplan et al., 1990; Lendahl et al., 1990). This expression persists until neuron differentiation, which triggers the expression of the neurofilament proteins (Shaw and Weber, 1982; Carden et al., 1987; Nixon and Shea, 1992); the presence of neurofilaments are indicators associated with mature neurons and thus increased expression of these proteins is concomitant with a decrease in the other development-associated intermediate filaments (Lariviere and Julien, 2004). NF-L expression is detected initially, at the beginning of neural differentiation and can regularly overlap with α-internexin and peripherin expression (Willard and Simon, 1983; Carden et al., 1897). Subsequently NF-M expression is correlated with neurite formation and NF-H expression occurs later in neuronal differentiation, during the postnatal period (reviewed in Larivere and Julien, 2004). The expression of intermediate filaments is not conserved in neurons and subpopulations of pyramidal neurons have been shown to express different intermediate filaments (Masliah et al., 1993; Dickson et al., 2000; 2005). Neurofilament expression is dramatically elevated after synapse formation, axonal diameter expansion and myelination (Nixon and Shea, 1992; Lee and Cleveland, 1996). The distribution of neurofilaments in mature neurons is not uniform; neurofilaments are generally more abundant in axons than dendrites and are more heavily phosphorylated in the axonal than somato-dendritic cellular compartment (Shaw and Weber, 1991).

The phosphorylation state of neurofilaments, and their interaction with the MAPs, governs the state of axonal integrity. The highly phosphorylated neurofilament present in axons allows the formation of side arms (reviewed in Lariviere and Julien, 2004) (Figure 1.5B). Side arms form a cross bridge with microtubules, other neurofilaments

and cortical F-actin, contributing to the structural characteristics of the axonal cytoskeleton. Not surprisingly, neurofilaments are particularly abundant in neurons with large diameter axons such as those of motor neurons where fast impulse conduction velocities are crucial for appropriate functioning. Additionally they are also involved in the regulation of axonal transport and the anchoring of cellular constituents (for review see Lariviere and Julien, 2004). However neurofilaments rarely enter the developmental growth cone, being contained to the axon and the axonal shaft. Furthermore, the presence of neurofilaments within the growth cone may be indicative of inappropriate growth cone function (King et al., 2001; Zhang et al., 2005).

Within the mature brain neurofilaments provide the structural framework required by neurons to maintain their polarised morphology. This axonal and dendritic polarisation is vital in creating the intricate network of fibres within the brain that transfers information in the form of electrical signals. The electrical connections of the brain is not maintained by the internal scaffolding alone, the surrounding glial cells within the brain play a vital role in the support and maintenance of neuronal function.

1.3 GLIAL CELLS OF THE MAMMALIAN BRAIN

Glial cells are the non-neuronal cells of the nervous systems and within the CNS include astrocytes, microglia, oligodendrocytes and ependymal cells (Figure 1.6). Glial cells are closely associated with neurons within the CNS (Figure 1.6) and have historically been understated in their roles within the brain. It was originally believed that glial cells played only a role in providing the scaffolding for neurons. However, many studies over the past decades have highlighted that glial cells are involved in a wide variety of functions within the brain and play many vital roles (Araque and Navarrete, 2010). Specifically, under normal conditions glial cells perform a variety of structural, nutritive and physiological functions including structurally supporting neurons, suppling vital nutrients and gases, insulating axons and facilitating action potentials, and destroying pathogens and the removal of debris (reviewed in Todd et al., 2006; Amor et al., 2010; Araque and Navarrete, 2010; Perea and Araque, 2010). Additionally during development, glial cells provided scaffold and guidance support to developing neurons.

1.3.1 Astrocytes

Astrocytes are the most common cell type in the CNS and there are approximately 10 times more astrocytes than neurons in the human brain (Bignami, 1982). Astrocytes are stellate-shaped cells with a highly ramified cytoskeleton and small cell body (three-five microns). There are two major populations of astrocytes; the protoplasmic astrocytes reside in the grey matter and the fibrous astrocytes reside in the white matter (Nolte, 2009). However, both types have essentially the same features, and both can be histologically identified by the expression of the astrocyte specific marker, glial fibrillary acidic protein (GFAP). Unlike neurons that interconnect through synapses, astrocytes are interconnected and communicate through gap junctions between cells. Astrocytes play a crucial role in structurally supporting neurons and the formation of the blood brain barrier. Astrocytes also play crucial roles in maintaining local ion concentrations and pH homeostasis in the extracellular space, assist neurons in the turnover of nutrients and metabolic by-products and neurotransmitters (glutamate), regulate synapse development and modulate synaptic strength (for reviews see

Nedergaard et al 2003; Haydon, 2001 and Seth and Koul, 2008). One such example of their other functions is glycogen metabolism. Astrocytes are the main source of glycogen in the CNS, which serves as an endogenous source of energy to both astrocytes themselves and the surrounding neurons (reviewed in Benarroch, 2010). Hence astrocytes are constantly interacting with neurons and contact is highly dynamic (Hirrlinger et al., 2004; Haber et al., 2006) and activity dependent (Genoud et al., 2006). One single astrocyte domain has the potential to contact hundreds of dendrites and up to 10000 synapses (Halassa et al., 2007; reviewed in Fellin, 2009).

1.3.2 Oligodendrocytes

Oligodendrocytes, another predominant type of glia in the CNS, have small cell bodies (one-three microns) and an elaborate array of branched projections (Nolte, 2009). Oligodendrocytes reside predominately in the white matter, which is rich in axons. The primary function of oligodendrocytes is to insulate axons, control axonal diameter and facilitate saltatory conduction with a specialised membrane sheath called myelin. Myelin wraps around axons, mainly large calibre pyramidal axons (Bradl and Lassmann, 2010); in fact they specifically select axons with a diameter of above 0.2 microns (Simons and Trajkovic, 2006). Myelin acts as an insulator to electrical signals travelling down the axon, thus increasing the speed of conductance. An individual oligodendrocyte can myelinate numerous axons, however each axon is myelinated by a single oligodendrocyte. The myelin sheath is not continuous down a single axon; between the myelin sheaths are gaps where the axon is exposed and action potential is regenerated in a saltatory manner, with these gaps termed nodes of Ranvier (Thaxton and Baht, 2010). These regions are dense in sodium voltage-gated ion channels (Waxman and Ritchie, 1993) and have an accumulation of axonal organelles (reviewed in Edgar and Nave, 2009). While their main purpose is myelination of axons, oligodendrocytes also play another supportive role for CNS neurons that involves the production of growth factors and guidance cues. Specifically they provide trophic support of the soma through the secretion of neurotrophins (reviewed in Tigue and Tripathi, 2008; Bradl and Lassmann, 2010). Furthermore, during development oligodendrocytes secrete inhibitory extracellular guidance cues that act to confine

developing neurites to correct trajectories (Nagashima et al., 1999; Goldberg and Barres, 2000), aiding in path finding.

1.3.3 Microglia

Resident microglia, which are the CNS-resident macrophages, when resting are smaller than astrocytes and oligodendrocytes and constitute approximately 5-12 percent of the total glial population (Nolte, 2009). Microglial cells are the primary immune cell type of the otherwise immune privileged CNS and are present throughout the CNS, however, generally the grey matter contains more microglia than the white matter (Rivest, 2009). Following entry into the CNS during embryonic, fetal and postnatal stages, microglial precursors undergo a defined sequence of developmental events involving dispersion and differentiation to generate a brain-resident population of macrophages (Cuadros and Navascués, 1998; Navascués et al., 2000). Once residing within the CNS, microglia develop a ramified morphology with active processes (Barron, 1995; Navascués et al., 2000). During brain development, microglia function as phagocytic cells that remodel the developing tissue (Barron, 1995). In the adult brain they participate in dynamic immune-surveillance functions (Nimmerjahn et al., 2005) activating in response to infectious organisms, brain injury and chronic disease (for a recent review see Rivest 2009).

Microglia, in conjunction with astrocytes and oligodendrocytes work closely together with neurons to achieve the highly integrated processing that occurs in the CNS. The complex interactions between all cell types allows fast and efficient transfer of electrical signals around the brain and to and from the brain stem and spinal cord. The complexity and specificity of connections renders the cells of the brain vulnerable to insults such as TBI.

1.4 THE RESPONSE OF THE MAMMALIAN BRAIN TO TBI

TBI causes trauma to a range of cells in and around the injury area. Accumulating evidence now indicates that rather than respond passively to brain injury, the brain

evokes an adaptive sequence of alterations directed at healing and remodelling (Maxwell et al., 1990; King et al., 2001; Oehmichen, 2004). Astrocytes, microglia/macrophages, oligodendrocytes, neurons, neural progenitor cells and brain vasculature all undergo adaptive alterations in response to a traumatic insult (Maxwell et al., 1990; Christman et al., 1997; Deller and Frotscher, 1997; Vickers et al., 2000; King et al., 2001; Finnie and Blumbergs, 2002; Tonchev et al., 2003; Lee et al., 2003; Lie et al., 2004; Emsley et al., 2005). Furthermore, inflammation is a common pathological consequence following injury, which disrupts the blood brain barrier (Lossinsky et al., 2004; Habgood et al., 2007) allowing cells from the blood stream to come in contact with the injury site. This post-traumatic inflammatory response can be characterised by glial activation, leukocyte recruitment, and upregulation and secretion of mediators such as cytokines and chemokines (reviewed in Ziebell et al., 2010). Understanding the coordinated response of the different cells types to injury is imperative in fully comprehending the brain's response to TBI.

The principal mechanisms causing brain injury can be divided into two broad categories: contact and non-contact injury (Finnie and Blumbergs, 2000). Contact injuries results from direct impact and may be penetrative, such as a gunshot wound, or non-penetrative, such as a direct strike to the head. Contact injuries additionally often generate local effects such as scalp laceration, skull fracture and extradural haematoma. Non-contact injuries are non-penetrative and result from dynamic inertial forces that rapidly rotate the brain within the skull, such as a rapid de-acceleration in a car accident. Both contact and non-contact injuries are capable of generating focal, multifocal and diffuse lesions (Povlishock and Katz, 2005). Focal lesions incorporate focal cortical contusion as well as both deep and extracerebral haemorrhage. In addition, shock waves generated at the site of impact may travel through the skull and brain, possibly causing contusions and intracerebral haemorrhage remote from the site of impact. Differential movement between the brain and the cranium may also generate diffuse injuries in which pressure gradients within the brain tissue create shear, tensile and compressive forces (Adams, 1995; Steward et al, 1999). Due to the combined influences of injury type and severity, and factors intrinsic to the patient, brain injury initiates an array of pathophysiological mechanisms.

The principal mechanisms evoked by TBI are classified as either primary or secondary damage. Primary damage following brain injury involves any neural disruption that is a direct result of the injury, occurring at the time of injury (Maxwell et al., 1997; Graham et al., 2000; Finnie and Blumbergs, 2002). Such damage may involve neuronal and glial cell populations as well as brain vasculature, inflammatory, neurochemical and metabolic alterations (Finnie and Blumbergs, 2002; Werner and Engelhard, 2007). In this regard, cells and blood vessels may be sheared, torn or stretched at the time of injury casing haemorrhage, axonal damage and immediate cell death (Finnie and Blumbergs, 2002). Primary injury initiates a progressive wave of secondary injury, exacerbating the original injury.

Secondary injury develops latently and can occur over a period of hours, days and weeks following the primary injury evoked by the initial trauma (Graham et al., 2000). Secondary perturbations include ischemia, excitotoxicity, spreading neurotoxicity due to disruption of ionic homeostasis and tissue destruction, energy failure, cerebral swelling, initiation of necrotic and apoptotic neuronal death cascades, neuronal degeneration due to downstream deafferentation/denervation and inflammation, which may in turn compound other responses and add to the spread of neurotoxic effects (Maxwell et al., 1997; Finnie and Blumbergs, 2002; Bayir et al., 2003; Chen et al., 2003a; Liou et al., 2003; Povlishock and Katz, 2005). Thus the delayed alterations following injury compounds the original effect of the primary injury resulting in further anatomical and functional deficits. For injury resulting from the direct insult there is limited scope for therapeutic intervention following injury. However the delayed clinical manifestations of secondary injury such as ischemia and hypertension are more malleable to therapeutic interventions.

1.4.1 The neuronal response to TBI

Studies from both experimental models of neuronal injury and cases of human TBI indicate that neurons undergo a stereotypical sequence of reactive and potentially regenerative alterations following injury (Maxwell et al., 1997). Neuronal alterations following injury are either a rapid result of primary injuries or a downstream effect of

secondary injury, with the time course ranging from hours to weeks. Changes in the soma may culminate in cell atrophy, death or survival (Kelly, 1981; Tuszynski and Gage, 1995; Martin, 2001; Singleton et al., 2002; Selzer, 2003). Due to their relatively long-distance projection and fragility, axons are particularly vulnerable to TBI, with axonal injury a common occurrence, even in seemingly mild cases of injury (Gennarelli, 1996; Maxwell et al., 1997; Smith et al, 2000).

1.4.1.1 Axonal injury

Axonal injury, either as a result of primary injury or a secondary effect, results in the formation of two axonal segments. Primary injury involves the direct physical disconnection of the axon into two separate segments. Disconnection of the axon as a result of secondary injury can be described as a nine-step process (Maxwell et al., 1997). Firstly perturbation of the axolemma occurs; secondly axon mitochondria swell; third involves the development of nodal blebs and decreased internode diameter; fourth is the loss of microtubules and changes in neurofilaments; fifth is an involution of the axolemma from the myelin sheath; causing, sixth, the formation of periaxonal spaces; seventh axon swelling occurs; eighth is the development of "myelin intrusions" and lastly the disconnection of the axon and the formation of axon bulbs, or "retraction balls". This process occurs at varying rates and may take several days to reach axotomy. Regardless of the mode of injury, following disconnection, the axonal ends almost immediately seal over and the proximal and distal sections begin to retract away from each other and enlarge. This is due to the blocked anterograde and retrograde transport of organelles and other material, including mitochondria, vesicles, multivesicular bodies and neurofilaments (Kelly, 1981; Di Giovanni, 2009). The distal segment becomes completely separated from the cell body of origin and ultimately degenerates by a mechanism that is known as Wallerian degeneration (Figure 1.7). The degeneration of the distal segment also results in a loss of synaptic input to the target cells. This leads to an alteration in synaptic activity in the post-synaptic cells, which can ultimately lead to dysfunction of that cell (Martin, 2001). Hence, a lesion at one site in the CNS can cause widespread disruption involving distant cells, according to the connections interrupted by the lesion. The persisting proximal segment of an axon has the ability to attempt to regenerate, or sprout (Figure 1.7).

1.4.1.2 Alterations in the cytoskeleton following injury

The neuronal cytoskeleton plays an important role in the neural response to both primary and secondary injury. Primary injury, in which axolemmal permeability is compromised, typically results in loss, accumulation or compaction of certain cytoskeletal components. However, as previously described, trauma without the disruption of axolemmal permeability can also cause cytoskeletal abnormalities (Pettus et al., 1994; Pettus and Povilshock, 1996; Maxwell et al., 1997 Graham et al., 2000).

While axons can recover from seemingly mild forms of compression, severe levels of compression can irreversibly block fast axonal transport due to mechanical breakage of the axoplasm (Gallant, 1992). Local reduction in the number of microtubules is a common consequence of axonal disruption (Hoffman et al., 1984; 1985; Jafari et al., 1997; 1998; Maxwell and Graham, 1997; Adlard et al., 2000), notably at the nodes of Ranvier (Maxwell and Graham, 1997), and may be accompanied by a loss MAPs such as tau and dendritic MAP-2 in the damaged neurons (Saatman et al., 1998; Zhang et al., 2000). Conversely the proximal stumps of severed axons have also been demonstrated to have an accumulation of tau (Smith et al., 1999). This loss of microtubules may be a result of a post-traumatic calcium influx, which favours microtubule dissolution (Graham et al., 2000). Disruption of microtubules and their associated motor proteins can disrupt the process of fast axonal transport, resulting in a failure of membrane-bound vesicles and cytoskeletal elements to be conveyed to distal axonal regions. However, this disruption may be only local, with fast transport continuing normally in regions where microtubules are intact, resulting in accumulations of cytoskeletal and other organelles at different sites along the damaged axon, contributing to multi-focal axonal swelling (Maxwell et al., 1997).

Neurofilament disruption following injury (Yaghmai and Povlishcok, 1992) can additionally have severe effects on neuronal structure and function (Posmantur et al., 2000), as neurofilaments provide mechanical strength to axons by counteracting compressive forces of the local environment (Hoffman et al, 1984; Cleveland et al., 1991). Neurofilament density is relatively constant over a wide range of axonal calibres

(Julien and Grosveld, 1991). Therefore larger axons will tend to contain a greater number of neurofilaments than smaller axons and respond in different ways when damaged, particularly when inflicted with tensile strain (Maxwell et al., 1997; Reeves et al., 2005).

Neurofilament compaction is a common consequence of injury (Okonkwo et al., 1998). Neurofilaments can persist in both proximal and distal axonal stumps for long periods, potentially due to the steric protection given by the remaining filamentous structure (Hall and Lee, 1995). This compaction can occur in both mild and severe forms of injury, and is dependent upon disruption of axolemma permeability (Jafari et al., 1997; 1998; Polvishock et al., 1997; Chung et al., 2005; DiLeonardi et al., 2010). Compaction can occur within minutes of injury and can persist for hours following the insult and is thought to be the result of loss and/or collapse of neurofilament side arms, with concomitant preservation of filamentous structure (reviewed by Maxwell et al., 1997). Neurofilament side-arm collapse following axonal injury may be the result of altered interaction between protein kinases and phosphatases. Alternatively, post-traumatic disruption of calcium homeostasis may abnormally activate proteolytic calpains, which cleave the neurofilament side arms from the core filamentous structure. Both processes could theoretically lead to a reduction in intra-filament spacing and therefore neurofilament compaction. It should be noted however, that even in the absence of disrupted axolemmal permeability and consequent neurofilament compaction, neurofilament disarray and misalignment could still occur (Maxwell, 1997).

Aberrant phosphorylation of neurofilaments is a hallmark of neurodegenerative diseases and injury (Petzold, 2005; Anderson et al., 2008; Staal et al., 2009; Shea et al., 2009). In the cell body and dendrites, serine/threonine phosphatases may maintain neurofilaments in a predominantly dephosphorylated form; while in the axon the phosphorylated neurofilament side-chains are maintained by protein kinases (Maxwell et al., 1997). After injury abnormally phosphorylated neurofilaments tend to accumulate in the cell body, while neurofilaments within the axons may become dephosphorylated. Neurofilament phosphorylation in the cell body impedes neurofilament transport into

the axon, subsequently contributing to decreased axon calibre following axonal damage (Schlaepfer, 1987).

Neurofilaments are present throughout the neurons except for the axon terminals. Calpains present in axon terminals are responsible for the breakdown of neurofilaments in this region; this degradation providing normal synaptic signalling feedback to the cell body (Schlaepfer 1987; King et al., 2000a). In the post-traumatic period, neurofilaments can be observed in distended axonal regions as well as infiltrating axon terminals, possibly indicating a disruption in calpain mediated neurofilament breakdown (King et al., 2000a). Support for this comes from *in vitro* (Dickson et al, 2000) and *in vivo* (King et al., 2000b; 2001) studies which have demonstrated extensive accumulations of neurofilaments into ring- and bulb-like structures in severed axonal stumps. Both structures lacked microtubules and appeared to contain a dense core of organelles (including mitochondria), surrounded by either a whorl (in ring-like structures) or dense ball (in bulb-like structures) of bundled neurofilaments. Interestingly, these damaged axons exhibit similar morphological changes to a subtype of dystrophic neurites characteristically observed in Alzheimer's disease (King et al., 2000b; Dickson et al., 2005).

The highly polarised nature of neurons renders them particularly vulnerable to TBI and axonal injury regularly occurs as a result of the insult. However it is not neurons alone that are affected during an injury. The surrounding glial cells are also susceptible to injury and they too are involved in the stereotypic response of the brain, undergoing an array of injury-induced alterations.

1.4.2 Response of glial cells to TBI

Glial cells undergo a variety of alterations following injury that can have both desirable and undesirable effects. Classically, reactive glial cells have been identified as the main inhibitor to regeneration following TBI. Indeed, the incapacity for axonal regeneration in the adult CNS can be attributed, at least in part to the limited ability of neurons to extend new processes in a non-permissive environment (Compston, 1995). Following injury, astrocytes, oligodendrocytes and microglia undertake active responses directed

at isolating and clearing the injured area. Specifically, astrocytes actively respond to injury, culminating in the formation of a glial scar, which acts to seal the injury site to prevent cytotoxic spread at the same time inhibiting neuronal regeneration (for a recent review see Sofroniew and Vinters 2010). Brain-resident microglia are also activated to transform into phagocytosing macrophages and along with macrophages derived from the circulatory system act to clear cell debris from the lesion site (Amor et al., 2010).

While astrocytes facilitate brain function in an uninjured brain, whether astrocytes play an overall beneficial or adverse function after brain injury remains unclear. Astrocytes undergo a stereotypical sequence of reactive alterations following TBI referred to as reactive gliosis. This process is characterised by cellular hypertrophy and hyperplasia, cytoplasmic enlargement, elongated cytoplasmic processes and an upregulation in the expression of GFAP (Maxwell et al., 1990; Ridet et al., 1997; Raivich et al., 1999; Kernie et al., 2001; McGraw et al., 2001; Chirumamilla et al., 2002; Chen et al., 2003a, b; Laird et al., 2008). Activated and enlarged astrocytes migrate to the lesion border and actively form the glial scar (Raivich et al., 1999; Kernie et al., 2001; Chen et al., 2003a, b; Laird et al., 2008). The glial scar is a dense structure comprised of these activated astrocytes and extracellular matrix proteins. It forms a physical barrier which acts to segregate the damaged from non-damaged tissue and may facilitate the isolation of the damaged area and removal of debris from the surrounding healthy tissue (Laird et al., 2008). Studies in transgenic mice in which reactive astrocytes were conditionally knocked out following experimentally induced injury demonstrated that the loss of reactive astrocytes results in a reduction in glial scar, which exacerbated the magnitude and duration of inflammatory activation with prolonged leukocyte infiltration, in comparison to non transgenic controls (Bush et al., 1999; Faulkner et al., 2004; Myer et al., 2006). Reactive astrocytes have also been implicated in other repair processes following injury including the facilitation of both blood brain barrier restoration and synaptogenesis (reviewed in Laird et al., 2008)

However, numerous studies have demonstrated that reactive astrocytes play inhibitory roles following injury as well (Fawcett and Asher, 1999). Classically, astrogliosis has been considered one of the fundamental extrinsic limitations to regeneration (Fawcett,

2006; Fitch and Silver 2008). Primarily the glial scar is impenetrable to regenerating neurites, making reconnection of the injured tissue impossible (Rieer and Houle, 1988; Silver and Miller, 2004). The reactive astrocytes within the scar release a range of extracellular matrix proteins that are inhibitory to axon growth, including the chondrotin sulphate proteoglycans, aggregan and versicam and neural-glial antigen-2 (Fawcett, 1992, 1997; Caroni, 1998; Fawcett and Asher, 1999; Mathews et al., 2002; Rhodes and Fawcett, 2004). Additionally, astrocytes play a crucial role in taking up extracellular glutamate in the uninjured brain. Following injury, astrocytes down regulate expression of glutamate transporters (Rao et al., 1998; Beschorner et al., 2007) leading to an increase in extracellular glutamate, which has excitotoxic effects in the injured brain (reviewed in Floyed and Lyeth, 2007). Reactive astrocytes are also implicated in the formation of brain edema following injury (Kimelberg, 1992) and the inflammatory response of the brain to injury, releasing a range of pro-inflammatory cytokines (reviewed in McGraw et al., 2001; Morganti-Kossmann et al., 2002; Laird et al., 2008).

Extended loss of oligodendrocytes can be a prominent feature of CNS injury (Emery et al., 1998; McTigue et al., 2001). Demyelination of axons, as a result of the oligodendrocyte loss, leads to dysfunction of axon potential conduction in the affected segments. As previously mentioned, oligodendrocytes may aid in path finding, confining developing neurites to correct trajectories (Nagashima et al., 1999; Goldberg and Barres, 2000). The oligodendrocyte guidance molecules that are present during development are inhibitory to axonal growth in the adult brain, which may be a factor in the inhibition of axonal regeneration following injury (Fawcett and Asher, 1999; Bandtlow and Schwab, 2000; Watkins and Barres, 2002; Grados-Munro and Fournier, 2003). Myelin plays a key role in the extrinsic inhibition to regeneration. CNS myelination is associated with cessation of axon growth (Nagashima et al., 1999) and loss of neuronal plasticity (Goldberg and Barres, 2000). Myelination may therefore provide growth inhibitory signals as the nervous system nears maturity. Furthermore, contact of regenerating neurites with either myelin or oligodendrocytes causes long lasting growth cone collapse, through intracellular signalling mechanisms (reviewed in Schwab et al., 1993; Filbin, 2003). Preventing oligodendrocyte development and the formation of myelin *in vivo* through local and repetitive X-irradiation induces enhanced

sprouting around the lesion site, in conjunction with persistent increased levels of the growth associated protein, GAP-43 (Kapfhammer and Schwabb 1994; Schwegler et al., 1995; Vanek et al., 1998). Oligodendrocytes have been linked to growth inhibition following injury due to the release of growth inhibitory factors, such as NogoA, myelin associate glycoprotein and oligodendrocyte myelin glycoprotein. Studies have demonstrated that these three proteins inhibit axon growth both *in vivo* and *in vitro*. Interestingly, all three proteins mediate their inhibitory activity through primarily the same receptor, Nogo-66. However, the myelination state alone does not appear sufficient in explaining the limited regenerative attempts. Studies have demonstrated that although central white matter substantially inhibits neuronal regeneration, regeneration is also inhibited by the relatively myelin-free CNS grey matter (Nagashima et al., 1999). Furthermore when the inhibitory myelin environment is removed completely while there is an increase in axonal outgrowth this rarely exceeds a few millimetres (Fawcett, 2006). Additionally, Davies et al. (1997) found that regenerating mature axons were able to extend for considerable distances in un-injured CNS white matter tracts. The PNS is capable of regeneration over relatively long distances, and differences between oligodendrocytes and Schwann cells, the myelinating cells of the PNS, may be attenuating the CNS regenerative capability (Watkins and Barres, 2002). Myelin within the PNS is rapidly removed following axonal injury. In contrast, this process takes considerably longer in the CNS, indicating that certain components of CNS myelin are likely to inhibit regeneration (Watkins and Barres, 2002). Additionally, in the PNS, myelin sheaths may not be disrupted, or myelinating cells may form new myelin conduits to guide and nurture regenerating axons (Kelly, 1981). This difference between myelinating cells ability to facilitate regeneration in the PNS in comparison to the CNS is due to intrinsic differences between PNS and CNS neurons and additionally differences in the myelinating cells themselves. However, while the myelin debris and myelin growth factors are inhibitory to growth in the injured brain, replacement of lost oligodendrocytes and remyelination following injury is required for proper neuronal functioning to be reinstated.

Microglia are activated in a range of neurodegenerative disease processes and also in response to brain injury (Ling et al., 2001; Kelley et al., 2007). Microglia are the main

cell type involved in the inflammatory response of the brain. In response to injury microglia migrate to sites of injury, multiply and develop into brain macrophages responsible for clearing neuronal debris and destroying pathogens (Thomas, 1992; Lotan and Schwartz, 1994; Aihara et al., 1995; Barron, 1995; Ravivich et al., 1999).

Classifying the response of glial cells to injury as either a desirable or adverse response remains under dispute. Indeed it is clear that a reactive response from the one cell type can evoke an array of effects. Specifically reactive astrocytes play a vital role in isolating and preventing the spread of neurotoxic damage following injury. Why this same structure prevents axonal regeneration remains to be determined. Similarly, guidance molecules secreted by oligodendrocytes inhibit axonal outgrowth. However, in a contrasting effect, remyelination following injury will be vital for proper axonal function. This intrinsic response of non-neuronal cells to prevent neuronal regeneration is curious as it indicates that induction of axonal growth following injury may not be functionally appropriate in the mature brain.

1.5 THE REGENERATIVE CAPACITY OF THE MATURE MAMMALIAN BRAIN

Full recovery following TBI will require the replacement of the dead and dying cells lost as a result of the injury, and either the appropriate extension of new axons to trace a path to their original target, mimicking the path finding abilities demonstrated by axons during development, or the compensatory remodelling of surrounding intact pathways to overcome the synaptic loss that occurred due to the injury. TBI does evoke an array of mechanisms within the injured neurons that are indicative of an attempt to regenerate and repair. Neurons respond to injury with a complex sequence of morphological, biochemical and gene expression alterations, some of which are directed towards the production of new neurons as well as sprouting, regeneration and synaptogenesis in resident injured neurons (Deller and Frotscher, 1997; Christman et al., 1997; King et al., 2000a; Magavi et al., 2000; Pastor et al., 2000; Kernie et al., 2001; Rice et al., 2003; Kerschensteiner et al., 2005). Injury can trigger the activation of precursor cell populations that in conjunction with resident cell proliferation have remarkable

potential to produce new neurons and glia in and around the injury site. Axons in the CNS often respond actively, rather than passively, to injury and undergo a distinctive sprouting response or regenerative attempt. The ultimate goal in this field of research is to facilitate full functionally recovery following TBI, which currently, cannot occur.

1.5.1 Neurogenesis and gliogenesis following injury

The notion that the mature brain is comprised only of post-mitotic, terminally differentiated cells and held in a rather static state, lacking the capacity to re-enter the cell cycle or regenerate following injury is no longer entirely valid. There is now convincing evidence that germinal regions within the CNS remain present in the adult brain, primarily the subventricular zone (SVZ) of the anterior lateral ventricles and the dentate gyrus of the hippocampus (Doetsch and Hen, 2005). The SVZ, in particular, is viewed as the principle germinal region of the adult brain and is comprised of neural progenitor cells capable of differentiating into neurons, astrocytes and oligodendrocytes (Reynolds and Weiss, 1992; Richards et al., 1992; Doetsch et al., 1997, 1999; Conover and Allen, 2002; Doetsch, 2003; Marshall et al., 2003; Mignone et al., 2004; Abrous et al., 2005; Doetsch and Hen, 2005).

Interestingly, it has now been demonstrated that trauma can alter the rate of cell proliferation in the mature brain. Alterations in progenitor populations and cell proliferation have been demonstrated in diverse models of experimental brain lesion and stroke conditions and are proposed to contribute to post-lesion brain recovery (Clarke et al., 1994; Duggul et al., 1997; Holmin et al., 1997; Kernie et al., 2001; Arvidsson et al., 2002; Chen et al., 2003a; Douen et al., 2004; Salman et al., 2004). Ultimately, injury-induced proliferation and progenitor cells may result in the generation of new neurons and/or glia (Gu et al., 2000; Magavi et al., 2000; Jin et al., 2001; Chen et al., 2003a, b; Douen et al., 2004; Salman et al., 2004). For example, experimentally induced ischemia can lead to the generation of new neurons in the injured neocortex (Gu et al., 2000). Furthermore, neural progenitor cells capable of extensive migration and integration have been demonstrated in several other brain regions (reviewed by Emsley et al., 2005). Studies have indicated that migrating neuroblasts, produced throughout life and largely destined for the olfactory bulb, may

be recruited to sites of brain injury (Ramaswamy et al., 2005). The extent of neurogenesis in the mature brain following injury remains to be fully determined.

Cellular proliferation following injury is not exclusive to neuronal progenitor populations, as gliogenesis has also been shown to increase following injury. For example resident microglia strongly increase their proliferation rate in response to injury (reviewed Rezaie and Male, 2002). Importantly astrogliogenesis has been shown to play a significant role in the formation of the glial scar (Tan et al., 2005; reviewed in White and Jakeman, 2008). While the presence of newly born astrocytes is correlated with traumatic insults to the brain, the origin of this astrogliogenesis remains under question. Thymidine analogue fate mapping experiments, in conjunction with retroviral and transgenic models to map cell fate, have demonstrated that the most likely origin of astrogliogenesis is NG2+ expressing glial precursor cells (Alonso, 2005; Burns et al., 2009). The role of NG2+ cells within the mature CNS is largely unknown; however it is clear that they greatly increase in number following traumatic insults (Dawson et al., 2000; Levine et al., 2001). NG2+ cells have the potential to generate a wide variety of cell types including astrocytes, but also oligodendrocytes and neurons, both *in vitro* and *in vivo* (Alonso, 2005; Belachew et al., 2003; Baracskay et al., 2007). Resident GFAP expressing astrocytes may also undergo proliferation in response to injury, these new astrocytes contributing to the glial scar (Buffo et al., 2008). It is important to note that the field of reactive astrocytes and the origin of gliogenesis following injury remain contentious. It is hard to determine whether an activated astrocyte within the injury site expressing precursor cell proteins is in fact a newly born astrocyte or a resident astrocyte reversing its differentiation state. This is due to the fact that studies have demonstrated that reactive astrocytes significantly alter the expression of a range of proteins, including the intermediate filament nestin, a filamentous protein classically associated with progenitor cells (Lin et al., 1995; Krum and Rosenstein, 1999; Douen et al., 2004). Thus is it hard to conclusively determine the origin of an activate astrocyte expressing the precursor protein nestin.

When damage results in extensive cell death the presence of newly born neurons may play a vital role in recovery of the brain following injury. Full functional recovery will

require the replacement of the dead and dying cells that were a consequence of a traumatic insult. Harnessing this intrinsic response of the resident progenitor cell population may provide a powerful tool to replace the neuronal loss that occurs in TBI.

1.5.2 Axonal sprouting following injury

Santiago Ramón Y Cajal first described the potential for neurons to respond to injury in the early 1900s, sketching reactive bulbs of injured neurite using light microscopy and silver staining techniques (DeFelipe and Jones, 1991). Numerous studies have now demonstrated an active response by damaged mature central CNS neurons, culminating in an attempt to regenerate (Salin et al., 1995; McKinney et al., 1997; Dickson et al., 2000; King et al., 2001; Bareyre et al., 2004; reviewed in Chuckowree et al., 2004). Frequently when the damaged mature neuron survives an insult the cell body and remaining portion of the injured axon undergo reactive changes involving the specific regulation of proteins, particularly cytoskeletal components, in preparation for an attempt at sprouting or regeneration (King et al., 2000a). Importantly, axonal regeneration and axonal sprouting are two distinct responses to axotomy, which may occur at the proximal stump of surviving injured axons. Regeneration implies successful restoration of the specific contacts between an axotomised neuron and its target, whereas sprouting describes the abortive response of the proximal stump to regenerate, leading to multiple local axonal collaterals without the restoration of normal connectivity (Berry et al., 1994; Deller and Frotscher, 1997). Many studies have now convincingly demonstrated that following injury surviving neurites have the ability to sprout axons into the local injury site (King et al., 2001; Chuckowree and Vickers, 2003; Haas, 2004).

Damaged axons in most regions of the mature CNS do not spontaneously regenerate and instead tend to undergo an abortive regenerative response restricted to the local neuropil (Cotman et al., 1994; Kapfhammer, 1997; Rhodes and Fawcett, 2004). The lack of full functional recovery is often attributed to the essentially inhibitory local environment of the mature brain (reviewed in Yiu and He, 2006) including the presence of myelin-associated inhibitors and the formation of the glial scar. However, as demonstrated in spinal cord injury, removal of the inhibitory CNS environment alone is

not sufficient in evoking full functional recovery (reviewed in Fawcett, 2006). Recent research indicates that there is an intrinsic loss of robust axonal extension. Whether the inability of the mature CNS to regenerate is due to the local non growth permissive environment, or to an inability to extend long axons which may be lost in a developmental switch (Li et al., 1995; Blackmore and Letourneau, 2006), or due to a switch from axonal to dendritic growth (Goldberg et al., 2002) or even a combination of all these factors, remains a priority research topic in the field of neuroscience. It is important to note however that the sprouting response into an injury site is not limited to directly injured neurons.

The restricted regenerative capacity of the mature CNS is seemingly not present in early development, being an acquired limitation. Long distance axon regeneration and full functional recovery following CNS injury has been displayed in embryonic models across species (Shimizu et al., 1990; Treherne et al., 1992; Hasan et al., 1993; Saunders et al., 1998). Both *in vivo* and *in vitro* models of perinatal healing and plasticity are indicative of mammalian cerebral cortical structures being capable of undergoing dramatic healing interactions and rapid new circuit reorganisation during the perinatal period. Comparison between regeneration in mature animals and younger animals highlights that growth occurring from regeneration in the mature CNS is shorter and less abundant (Varga et al., 1995). This capacity for greater regeneration is believed to be lost during a discrete developmental transition. The reduction of regenerative capacity occurs a few weeks postnatally in rodents and a few months in humans (Chen et al., 2002).

A key event in axonal survival and the potential for regeneration is a substantial reorganisation of the cytoskeleton (Christman et al., 1997) and the formation of an axonal growth cone (Spira et al., 2003; Erez et al., 2008). While the cellular mechanisms that govern the transformation of a severed axon into a growth cone have been extensively investigated (Ashery et al., 1996; Spira et al., 2003; Sahly et al., 2003; Erez and Spira, 2008) the precise mechanisms underlying this transformation remains unclear. What is clear however, in *Aplysia* neurons, is the formation of regenerative growth cones is governed by the level of intracellular calcium (Kamber et al., 2009). If

the calcium level is too low the injured neurite forms an end bulb whereas when calcium reaches adequate levels, the stump forms a growth cone (Kamber et al., 2009). Local protein synthesis at the distal portion of the proximal axon enables these intrinsic triggers to form a regenerative cone (Twiss and Minnen, 2006). Neurofilaments may also play a crucial role in determining whether and how central neurons regenerate following axon transection (Hall and Yao, 2000) as indicated by the disrupted regeneration exhibited by neurofilament-deficient mice (Zhu et al., 1997) and quail (Jiang et al., 1996) following peripheral nerve injury. However, sprouting axons around the injury site rarely result in a true regenerative attempt and the formation of correct synapses (Neumann and Wolf, 1999; Neumann et al., 2002; Hammarlund et al., 2009). Enhancing the regenerative response is a key goal in many studies, however, any modification that results in an extensive and functionally appropriate sprouting response still remains to be elucidated.

A prominent theme in current research in neuroscience is based upon facilitating and promoting axonal regeneration following brain injury. However, still little is known about the accuracy of these regenerating sprouts. Sprouting around the injury site may not always be beneficial or desirable. Both *in vitro* (McKinney et al., 1997) and *in vivo* (Salin et al., 1995) experimental models indicate that post-injury sprouting may not always be functionally appropriate, with potential aberrant axonal connectivity possibly contributing to the development of epilepsy following brain traumas such as stroke (Carmichael, 2003). Indeed many of the new connections that form when damaged axons sprout and re-synapse with targets are hyperexcitable (McKinney et al., 1997) and traumatic epilepsy is a common consequence of traumatic head injury (Willmore, 1995; Jacobs et al., 2000; Santhakumar et al., 2001). The onset of epileptic seizures typically occurs one to three years after head injury and the likelihood of seizure evolution is correlated with the severity of the initial injury (Willmore, 1995). Additionally, post-lesion sprouting has also been implicated in the generation of abnormal circuitry in other brain lesion paradigms (Jacobs et al., 2000; Parent, 2002; Parent and Lowenstein, 2002). Thus the induction of growth, after injury, may be inappropriate, potentially exacerbating a clinically poor outcome. There is a current interest in rehabilitation in conjunction with induction of axonal growth following

injury as a TBI intervention. Interestingly, rehabilitation has the potential to reinforce appropriate synapses and induce the dieback of inappropriate connections (reviewed in Fawcett, 2009). This therapeutic intervention may provide a non-invasive mechanism to eliminate aberrant sprouting. Induction of appropriate axonal sprouting of injured and collateral axons, synaptogenesis and a generalised plasticity will be of vital importance in promoting functional recovery from injury to the CNS. Clearly any intervention that prevents post-traumatic neurodegeneration, or inappropriate axonal sprouting and enhances adaptive neural responses has the potential to substantially improve the prognosis of TBI. A great deal of emphasis is therefore being placed upon developing strategies to promote neural repair following injury. However, many of the basic mechanisms underlying endogenous repair mechanism are not fully understood and need to be discovered to prevent adverse outcomes.

1.5.3 Potential for structural plasticity in mature mammalian brain following injury

The current body of research emerging indicates a remarkable plasticity retained within the brain which, until recently was thought not to exist. The cental dogma that the mature brain is a fixed, undynamic structure is no longer valid. The advancement of multi-photon microscopy techniques in the past decade (Denk et al., 1990) has convincingly demonstrated that the mature brain does retain a capacity for remodelling and plasticity. Collateral sprouting from uninjured axons also play a large role following axonal injury (Salin et al., 1995; Deller and Frotscher, 1997; Jin et al., 2006), possibly due to availability of vacant synaptic territory within the injury site (McKinney et al., 1997). However if the injury has resulted in significant cell death, collateral sprouting cannot account for the lost synaptic connections alone, replacement of the lost cells must first occur.

Under normal physiological conditions the mature brain is a dynamic network of connections that are constantly remodelling and responding to experience-dependent learning. This day to day plasticity is predominantly in dendritic spines which undergo rapid turnover through extension and retraction and alterations in their shapes and sizes (for a selection of recent papers see Grutzendler et al., 2002; Knott et al., 2002;

Trachtenberg et al., 2002; Majewska and Sur, 2003; Portera-Cailliau et al., 2003; Holtmaat et al., 2005; Majewska et al., 2006; Brown et al., 2007; Brown et al., 2008; Lee et al., 2008; Brown et al., 2010); which has been associated with synapse formation and elimination (Trachtenberg et al., 2002; Holtmat et al., 2008). Additionally, studies have shown a remarkable extension and retraction of dendrite tips within subpopulations of neurons in the naïve brain (Lee et al., 2006; 2008).

Alterations in synaptic strength and wiring, remodelling of axonal and dendritic arbors and changes in dendritic spine and axonal bouton turnover, have been observed not only in the naïve brain but also in response to injury. Cortical neurons demonstrate axonal and synaptic remodelling in response to various models of injury (for examples see King et al., 2001; Knott et al., 2002; Trachtenberg et al., 2002; Majewska et al., 2006). Reorganisation such as this may correlate with functional recovery not only in stroke (Carmichael, 2003; Carmichael, 2006; Brown, 2008; De Filippo et al., 2008), but comparable plasticity may also account for the recovery observed after forms of spinal cord injury (reviewed in Edgerton, 2004; Dunlop, 2008). Experimentally induced complete infarction of the somatosensory cortex induced a new pattern of electrical circuit activity in the intact ipsilateral hemisphere (Takatsuru et al., 2009). Compensatory plasticity can involve axonal sprouting of intact neurites and synapse alterations or the growth of new horizontal connections. However another early plastic mechanism evoked by injury is the unmasking of silent pathways in the affected network and can occur within hours of a nerve lesion (Merzenich et al., 1983; Donoghue et al., 1990). Spontaneous reorganisation in small cortical lesions results in adjacent cortical areas taking over the function of the damaged areas (Nudo, 1999; Kolb, 2003). This activation of functionally inactive connection occurs through an increase in excitatory transmitter release, an increase in density of postsynaptic receptors and changes in membrane conductance in conjunction with a decrease in inhibitory input or removal of inhibition from excitatory input. Plasticity can also occur over a longer time period, evoked through different mechanisms. Long-term changes occur through long-term potentiation and depression, and, evidenced in the motor cortex, involve synaptic alterations in N-methyl-D-aspartic acid receptor activation and an increase in intracellular calcium concentration (Hess and Donoghue, 1994; 1996).

When damage to a circuit is relatively small, it is feasible that reorganisation of the surrounding networks can recover the functional loss, however complete destruction within an injury site will require the replacement of lost and dying cells and frank extension of lost axons. The specificity and degree of remodelling that can occur in response to injury has not been fully elucidated. To understand how to manipulate both the uninjured and injured brain's potential for plasticity to facility a functionally desirable outcome following injury, the full potential and plastic response of neurons to injury must first be fully understood.

1.6 THESIS AIMS

Despite an accumulating body of literature regarding the capacity for neural remodelling and regeneration following structural brain injury many aspects of this response remain to be fully elucidated. This thesis investigated the reactive and regenerative alterations associated with the neural response to physical injury in the adult mammalian brain. The studies to be included utilised a range of *in vitro* and *in vivo* models of primary injury. To investigate the regeneration potential of the mature mammalian brain a variety of procedures were utilised, including immunohistochemical, molecular and imaging techniques, to examine the morphological, cellular and molecular events that characterise the neural response to injury. This thesis studies are focused upon the potential for regeneration following injury. Specifically, how comparable regenerating neurons are to their developmental counterparts, characterising alterations within the damaged neurons and the surrounding brain following injury, and the role of the neuronal cytoskeleton in neuronal regenerative events, as the mechanisms underlying these processes are currently poorly understood.

Aim 1: To determine the motile characteristics which govern axotomised CNS neurons during regeneration and how these changes compare to initial neurite development. Axonal outgrowth and pathway selection plays a crucial role in development of the mammalian brain and will thus be important in axonal sprouting and/or regeneration. The first aim addressed by this thesis endeavoured to characterise the motile capabilities of regenerating axons in comparison to development *in vitro.* It utilised an established model of axonal transection of rat cortical neurons, grown to relative maturity. The response of developing and regenerating axons was analysed using multi-labelling immunocytochemistry and live cell imaging.

Aim 2: To characterise the response and regenerative attempt of injured neurons to a novel model in injury *in vitro* and determine what degree the injury response is dependent upon mode of injury. Due to the wide varieties in injury that can occur

as a result of a TBI, full comprehension of the response of the brain to various modes of injuries and mechanisms underlying these responses will be vital in devising successful treatment. Thus the current aim endeavoured to develop an *in vitro* model of axonal injury that involved a total shearing of axonal process from the neuronal cell body, to determine how much the neuronal regenerative response relied upon mode and degree of injury. The ability of relatively mature neurons to survive and attempt a regenerative response after this novel model of axonal shearing was investigated. The response of the neurons to this mode of axonal injury, and in comparison to the transection model of injury utilised in Aim 1 was examined using a combination of live imaging and immunocytochemical analysis.

Aim 3. To investigate the reactive and regenerative changes that characterise the neural populations response to injury *in vivo*, exploring the co-ordinated alterations, in both neuronal and glial cell populations, which occur following injury. The extent to which the adult brain undergoes recovery following injury remains controversial. A rodent model of structural injury specific to the neocortical grey matter of the somatosensory cortex was utilised. These studies aimed to determine the ultimate fate of injury-induced proliferating and progenitor cells and the potential for these cells to contribute towards neuronal replacement in the injured adult neocortex. Furthermore the response of various populations of neural cells, including neurons, astrocytes, microglia and macrophages as well as alterations in brain vasculature, was analysed with immunohistochemical techniques, focussing specifically upon the neuronal cell type specific response to injury *in vivo*.

Aim 4. To characterise the phenotype of neurons derived from a transgenic mouse lacking the neurofilament light chain protein and determine the regenerative abilities of the neurofilament light chain null neurons. The axonal cytoskeleton, including the neurofilaments, plays a crucial role in both initial neurite outgrowth and elongation and regeneration. This aim examines the cytoskeletal alterations, specifically the reactive and regenerative changes that characterise the axonal response to physical injury in neurons lacking the neurofilament triplet protein NF-L. To meet this aim, a novel *in vitro* model of axonal injury derived from transgenic mice with a NF-L deletion was utilised, in conjunction with the focal acute neocortical injury

utilised in Aim 3. The neuronal characteristics and response of regenerating axons was analysed using multi-labelling immunohistochemistry, immunocytochemistry and molecular techniques.

This thesis endeavoured to characterise the reactive and regenerative response of mature CNS neurons to injury. These studies included provide insight in to some of the aspects of the post-injury neuronal response and further explain why the mature CNS is incapable of repair. Results from theses studies enhance current knowledge regarding the basic mechanisms underlying neuronal and axonal regeneration and highlight certain neuronal responses to injury in which therapeutic intervention may be possible.

2 MATERIALS AND METHODS

2.1 CORTICAL NEURON CULTURE

2.1.1 Preparation of coverslips and cell culture trays

Glass coverslips were nitric acid etched through submersion in nitric acid overnight, followed by 5 milli Q® washes. Coverslips were then oven dried at 80°C and autoclaved. Coverslips were singularly placed into either 12-well or 24-well microplates (Iwaki, Japan) within a laminar flow hood and further sterilised through ultraviolet radiation for 30 mins. 1mg/mL poly L-lysine (Sigma, USA) in borate buffer, pH 7.4 was filter sterilised onto the coverslips using Acrodisc® 25 mm syringe filters and left for 24 hrs. After this time the poly-L-lysine was removed from the coverslips and 1mL of Neurobasal^{TM} media supplemented with 2% B27, 10% foetal calf serum, (all from Invitrogen, USA) 0.5mM L-glutamine, 25μm glutamate and penicillin/streptomycin antibiotics (all from Gibco BRL, Life Technologies, USA) at 37°C was added. The coverslips were then incubated at 37°C in 5% CO_2, until plating of culture.

2.1.2 Dissection and dissociation of cortical tissue

The Animal Experimentation Ethics Committee of the University of Tasmania approved all procedures involving animals.

Primary dissociated cortical neuron cultures were prepared from embryonic day 18 (E18) Hooded-Wistar rats under sterile conditions as previously described (Banker and Goslin, 1988). Rats were euthanased through CO_2 asphyxiation at 7 L/min for 6 mins. Each rat is checked for reflexes and the site of incision alcohol sterilised. An incision was made along the midline of the stomach and the embryos removed and placed on ice. Within the laminar flow hood, the embryos were removed from the amniotic sac and decapitated. The outer layers of the skull and meninges were dissected and the superficial layers of the frontal cortex of both hemispheres removed and placed in 5mL of 10mM HEPES at 37°C.

Following the complete dissection of all embryos, 0.25mL of 0.025% trypsin (Sigma, USA) was added to the 10mM HEPES and cortical tissue and incubated in a water bath at 37°C for 10 mins. Following this, all solution was carefully removed, and 5mL of fresh 10mM HEPES added. The tissue was then incubated in the water bath at 37°C for five mins, this washing procedure repeated twice. After the final wash 5mL of fresh 10mM HEPES was added and cell dissociation was completed through gentle trituration using a 1mL pipette. Cell viability was assessed using trypan blue vital dye exclusion (Sigma, USA). Cells were plated onto the prepared coverslips, to a density of $5.0x10^5$ for the 24-well plates and a density of $1.0x10^6$ for the 12-well plates. The plates were then returned to the incubator.

2.1.3 Maintenance

Two days after plating, the Neurobasal™ media containing 10% foetal calf serum was removed and replaced with 2ml of subsequent Neurobasal™ media supplemented with 2% B27, 0.5mM L-glutamine and penicillin/streptomycin antibiotics to selectively promote the growth of neurites (Brewer et al., 1993, Brewer 1995; 1997). Every three to four days half of the media was removed and replaced with fresh media. Cells were maintained in the 37°C humidified atmosphere of CO_2 for up to 24 days *in vitro* (DIV).

2.2 *IN VITRO* AXONAL INJURY

Axonal injury was carried out as previously described in Dickson et al., (2000) and Chuckowree and Vickers, (2003) on mature cortical culture. Cell culture maturity is reached at 21 DIV, when a lack of expression of developmental proteins (Chuckowree and Vickers, 2003; Haas *et al*., 2004) coincides with the expression of proteins correlating to maturity of the neuron and its processes (Chuckowree and Vickers, 2003; King *et al*., 2006). At 20 DIV, coverslips were transferred to individual 35 mm sterile plastic petri dishes (Iwaki, Japan) within the laminar flow hood and allowed to re-acclimatise to the incubator conditions for 24 hrs. At 21 DIV, individual axonal bundles interconnecting discrete neuronal aggregates adherent to underlying substrate were selected for transection. Transection was performed under microscope guidance using a 12cm Barkan goniotomy curved-blade diamond knife (Kaisers, Germany). A

transection was made with the knife blade on appropriate axonal bundles to completely transect axons and leave a cell free lesion. Several injuries (2-5) were made per coverslip.

2.3 *IN VIVO* FOCAL NEORCORTICAL INJURY

Acute focal neocortical injuries were performed as previously described (King et al. 1997, 2001; Dickson et al. 2005). Briefly, following intraperitoneal administration of anaesthetic (pentobarbitone sodium, 72mg/kg, Abbot Laboratories, Australia) and analgesic (Carprogen, 4mg/kg, Pfizer, Australia), animals were immobilised in a Stoelting stereotaxic frame. A burr hole was drilled into the skull, (5mm anterior and 4.5mm lateral to lambda for rat, 2.5mm anterior and 2mm lateral to lambda for mice) and a focal injury was made in the somatosensory cortex (Par1 region) by lowering a 25-gauge Hamilton needle (Reno, USA) to a depth of 1.5mm into the grey matter for rats and a 29-gauge needle to a depth of 1mm into the grey matter for mice. The needle was left in place for ten minutes prior to removal and suturing of the wound.

2.4 IMMUNOCYTOCHEMISTRY AND IMMUNOHISTOCHEMISTRY

Fixed cultures and brain sections were incubated in combinations of mouse and rabbit primary antibodies in diluent (0.3% Triton X-100 in 0.01 M phosphate buffered saline (PBS)) for two-hours at room temperature on a shaker followed by incubation at 4°C overnight (16-20 hr). Primary antibodies were then removed through three ten-minute washes in 0.01 M PBS. Secondary antibodies goat anti-mouse Alexa Fluor® 594 and goat anti-rabbit Alexa Fluor® 488 (dilution 1:1000; Molecular probes, USA) were diluted in 0.01 M PBS and incubated on coverslips for 90 mins on a shaker in the dark. Nuclear yellow (Molecular Probes, USA, 0.01%) was added for the final 5 min of the secondary antibody incubation, prior to washing and mounting. Coverslips and sections were then washed three times in 0.01 M PBS and mounted on to glass slides using Permaflour aqueous mounting medium (Immunotech, France). Immunoreactivity visualised using a Leica (Germany) DM BL2 upright fluorescent microscope.

Table 2.1 Primary antibodies used for immunohistochemistry

Antibody name	Species	Immunoreactivity	Dilution	Supplier
α-internexin	R	Class IV intermediate filament subunit	1:2000	Novus Biologicals
β-III-tubulin	M	Neuron specific β-tubulin subunit	1:10000	Promega
BrdU	M	Bromodeoxyuridine, thymidine analogue	1:1000	Sigma
Calretinin	R	Interneuron calcium binding protein	1:2000	Chemicon
ERM	M IgM	Actin/membrane binding exrin-radixin-moesin protein	1:500	Gift from F. Solomon
Ferritin	R	Microglial iron binding protein	1:2000	Dako
GFAP	R	Glial fibrillary acidic protein	1:2000	Dako
Ferritin	R	Proliferating cell nuclear antigen	1:500	Zymed
Ndel1	R	Mammalian NudE protein	1:100	Gift from D. Smith
Nestin	M	Intermediate filament protein marker of neural progenitor	1:1000	BD Biosciences
NF-L	R	68kD neurofilament triplet light molecular weight subunit	1:1000	Millipore
NF-M	R	150kD neurofilament triplet medium molecular weight subunit	1:2000	Serotec
Parvalbumin	MC	Interneuron calcium binding protein	1:2000	Swant
PCNA	R	Proliferating cell nuclear antigen	1:500	Zymed
SMI312	MC	Pan-axonal neurofilament marker	1:2000	Swant
Tau	R	Microtubule associated protein	1:15000	DAKO
Trk B	R	Tyrosine kinase receptor B	1:500	Santa Cruz

R = Rabbit polyclonal antibody, M = Mouse monoclonal antibody, MC = Mouse monoclonal cocktail. Antibodies are IgG unless otherwise specified.

2.5 LIVE CELL IMAGING

Time lapse imaging was carried out using differential interference contrast (Normarski) microscopy on a Leica (Germany) DM IRB inverted microscope. Selected coverslips were transferred to a mounted 0.17mm Delta T dish (Bioptechs, USA) containing imaging buffer (in mM: 124 NaCl, 5 KCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 30 dextrose and 24 HEPES, pH 7.3) (Zhang and Benson, 2001, Chuckowree and Vickers, 2003) at 36°C, CO_2 enriched. Temperature inside the dish was maintained through a Delta T4 culture dish controller (Bioptechs, USA). Imaging buffer underwent constant replenishment from a stock of imaging buffer, maintained at 37°C with carbogen (5% CO_2 in oxygen; BOC gases, Australia) being pumped through at 3 L/min. Imaging buffer was replaced and removed from the Delta T dish at a constant rate. Preliminary trials determined the optimal rate of imaging buffer replenishment and carbogen enrichment. Appropriate cells clusters were randomly selected which were free from contact with surrounding neuronal cells clusters and glial populations. A standard minimum distance of 50μm from the selected axon and surrounding cells was set.

Live cell images were captured every minute over a two-hour period with a Hamamatsu Orca ER (Hamamatsu Photonics, Japan) digital camera attached to a G4 Macintosh computer (Apple Computers, USA) using the Open Lab 4.0.2 program. To ensure that the intrinsic motility of axons was the variable investigated, all external variables of growth cone and axonal motility were controlled. The imaging environment constructed was completely devoid of external stimuli except for a constant light source. All cells included in the data were imaged once; stimulus history of growth cone *in vitro* can alter behavioural response (Diefenbach et al., 2000). The strict regulation of content of imaging buffer and replenishment rate ensured calcium levels (0.02mM $CaCl_2$) were kept at a controlled physiological level.

3 CELLULAR DYNAMICS UNDERLYING REGENERATION OF DAMAGED AXONS DIFFERS FROM INITIAL AXON DEVELOPMENT

3.1 INTRODUCTION

While the mature brain, unlike the immature CNS and mature PNS, cannot completely repair following injury, it is now clear that the mature brain evokes an active response to injury and that this response culminates in an attempt to regenerate (Salin et al., 1995; McKinney et al., 1997; Dickson et al., 2000; King et al., 2001; Bareyre et al., 2004; reviewed in Chuckowree et al., 2004). The lack of full functional recovery is often attributed to the essentially inhibitory local environment of the mature brain (reviewed in Yiu and He, 2006) including the presence of myelin-associated inhibitors and the formation of the glial scar. However, experimental evidence has convincingly demonstrated that removal of the inhibitory environment alone is not sufficient in evoking full functional recovery (reviewed in Fawcett, 2006), thus indicating that there must also be an intrinsic limitation to full functional recovery. Understanding the intrinsic limitation to mature CNS regeneration will be vital in devising effective strategies to treat TBI.

Recent studies have indicated that the lack of robust axonal extension following injury may be an intrinsic limitation. It has been postulated that this ability to extend long axons may be lost in a developmental switch (Li et al., 1995; Blackmore and Letourneau, 2006). Following TBI, to re-establish the lost connections, thus reinstating the neuronal network, an injured axon must robustly extend into the injured environment and navigate its way to the correct, original target. During development, neurons become polarised and their axons extend relatively long distances correctly tracing a path to their ultimate target. Critical to this precise and accurate axonal outgrowth that occurs during brain development is the growth cone. These transient structures, present on the tips of elongating axons, have both sensory and motor capabilities, which enable the integration of environmental cues, such as growth factors, resulting in precise path finding, ultimately culminating in the establishment of connectivity in extensive neuronal networks (for review see Tessier-Lavigne and

Goodman, 1996; Suter and Forscher, 2000; Gordon-Weeks, 2004). During development growth cone form is behaviour specific (Mason and Wang, 1997; Szebenyi et al., 1998), with these structures exhibiting striking changes in their morphology in different cellular contexts.

Aspects of the neuronal regenerative response recapitulate the sequence of dynamic cellular and morphological features displayed by developing neurites (reviewed in Harel and Strittmatter, 2006). Numerous studies (eg Hoffman et al., 1987, 1993; Goldstein et al., 1988; Hoffman and Cleveland, 1988; Muma et al, 1990; Nixon and Shea, 1992) have shown that following axonal injury and during regenerative attempts, neurofilament expression is down-regulated, whereas expression of other cytoskeletal elements is elevated, suggesting that the sequence of cytoskeletal gene expression occurring in development is recapitulated during attempted regeneration (Hoffman and Cleveland, 1988; Lee and Cleveland, 1996). It has be demonstrated, both *in vitro* (Dickson et al., 2000) and *in vivo* (Christman et al., 1997; King et al., 2001), that the regenerative attempt mounted by axons is associated with cytoskeletal reorganisation and accompanied by an up-regulation of GAP-43. GAP-43, a neuron specific growth and membrane-associated phosphoprotein, is one of the most abundant proteins in neuronal growth cones, where it is implicated in filopodial motility, axon guidance, synaptogenesis and synaptic plasticity (Brandt, 1998). It is, therefore, not surprising that the expression of GAP-43 is down-regulated in the mature CNS, but elevated during both neuronal development and regeneration (Goslin et al., 1988; Cantallops and Routtenberg, 1999).

However, observations from fixed preparations of focally damaged neocortex, suggest that regenerating axons also have a number of features that differ from developing neurites, such as a high intra-axonal density of neurofilaments and club-like endings on distal regenerating tips (King et al., 2001). Similarly, live imaging of acute slices revealed that although regenerative sprouts are highly motile, they differ substantially in morphology to classical developmental axonal growth cones, being relatively simple finger-like structures (Dickson et al., 2007). Similarly, post-injury regenerating axons described in the adult lamprey CNS, are neurofilament-rich, relatively simple in

structure and lacking filamentous actin at the sprouting tip (Zhang et al., 2005). The similarities and differences between regenerating and developing axons are still under investigation. While the cytoskeletal composition, form and behaviour of growth cones during development has been intensively investigated (Kalil et al., 2000; Dent and Gertler, 2003; Zhou and Cohan, 2004), comparatively, little is known about the morphology, pathway selection and biochemistry of the tips of regenerating axons.

This Chapter investigated the cytoskeletal and dynamic characteristics of mature regenerating axons compared to developing axons *in vitro*, endevouring to classify the similarities or differences that may be present between regenerating and developing axons. It involved the direct comparison of axons and growth cones *in vitro* during development and after injury, the latter ultilising an *in vitro* neuronal transection injury model (Dickson et al., 2000; Chuckowree and Vickers, 2003; Chung et al., 2003). Neurite transection was performed at 21 DIV, when neurons are relatively mature. These neurons are considered relatively mature due to the absence of developmental markers such as GAP-43 (Dickson et al., 2000) and the Ezrin-Radixin-Moesin family of membrane-cytoskeletal linker proteins (Haas et al., 2004), and the presence of markers indicative of neuronal maturity such as punctate synaptic proteins (mature N-methyl D-aspartate receptors and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors localized to dendritic spines) microtubule associated protein 2 (MAP-2) and the neurofilament triplet proteins (for example, Chuckowree and Vickers, 2003; King et al; 2006). Immunolabelling for cytoskeletal markers were used to compare growth cones on regenerating axons to growth cones at comparable time-points during development *in vitro*. The behaviour of growth cones during regeneration and development was also investigated utilising live imaging time-lapse microscopy.

3.2 MATERIALS AND METHODS

3.2.1 Neuronal culture and *in vitro* model of axonal transection

Primary dissociated cortical cultures were prepared as previously described (Chapter 2.1)

Axonal transection was carried out as described in Chapter 2.2 on developmental cultures at 5 DIV and on relatively mature cultures (21 DIV). The sprouting response observed in cortical culture *in vitro* has been shown to commence between 4 to 6 hours (hrs) post-injury (PI) (Chuckowree and Vickers, 2003). Therefore the imaging time-point, 6hrs PI, represents the initial sprouting response of the transected axons, whereas analysis at 24hrs PI allows the examination of the sustained sprouting response.

3.2.2 Growth factor application

Two growth factors were selected on the basis of their well-established ability to enhance neurite outgrowth during development (Tucker et al., 2001, Airaksinen and Saarma, 2002, Goldberg et al., 2002) and affect terminal axonal sprouting in response to injury (Zhou and Shine, 2003; Hafidi et al., 2004) in other neuronal populations. Human recombinant Brain Derived Neurotrophic Factor (BDNF) (20ng/mL, reconstituted in milli Q®) (Chemicon, Temecula, CA) or Glia Derived Neurotrophic Factor (GDNF) (20ng/mL, reconstituted in milli Q®) (Chemicon, Temecula, CA) and vehicle controls (milli Q®) were bath applied to cultures at 3, 5 and 7 DIV and immediately following transection at 21 DIV for 16 hrs. Concentrations were based on preliminary investigations determining maximal effect on outgrowth, with application of an increased concentration (50ng/mL) not associated with enhanced neurite growth (data not shown).

3.2.3 Immunocytochemistry

Cultures (3, 5 and 7 DIV and 6 and 24 hrs post-injury in 21 DIV cultures) were fixed in 4% paraformaldehyde (PFA)/0.01M PBS for 30 mins at room temperature on an orbital shaker. Coverslips were then processed for immunocytochemistry as described in

Chapter 2.4 (for antibody details see Table 2.1). To label for filamentous actin (F-actin), cultures were incubated with AlexaFluor 594 phalloidin (dilution 1:200; Molecular Probes), for 30 min after immunocytochemistry.

3.2.4 Live Cell Imaging and Analysis

Live cell imaging was carried out as described in Chapter 2.4. For the post-injury investigations, images immediately prior to injury were captured and referred to at the later time-points to ensure the axons under investigation were severed. Axonal extension and growth cone motility over the 2 hr imaging time period was quantitated using the individual time-lapse images. Twenty-five 2 hr time-lapse images were included in the analysis, 5 images for each time-point from separate cultures. To analyse the dynamic movement of the individual axons the centre of the growth cone of the axon under investigation was assigned an X, Y co-ordinate every 2 mins (n=25). The original position of the growth cone, at the time-point 0, was assigned the X, Y co-ordinate 0,0. The original X, Y position was then subtracted from subsequent X, Y co-ordinates. Mean axonal extension was calculated by determining axonal length over the two-hours, measured at 10 min intervals. Growth cone behaviour was analysed every minute (n=25) and classified as extending, pausing or retracting, calculated as a percentage of the total time imaged. For growth factor application experiments, neurite length was measured using OpenLab software. *t* tests or ANOVA were performed on quantitative data, with *post hoc* comparisons (Bonferroni test) as appropriate using Prism v4.0 software (Graphpad, USA).

3.3 RESULTS

3.3.1 Regenerating cortical neurons have a similar cytoskeletal profile to developing neurons in vitro

Immunocytochemistry was performed at 5 DIV and following transection at 6 hrs post-injury (Figure 3.1). Immunolabelling with antibodies to both tau (an axonal marker) and MAP-2 (a somato-dendritic marker) was used to confirm the axonal identity of the neurites under investigation (Figure 3.1A,B,E and F). Tau and MAP-2 were co-localised within the cell bodies of developing neurons, with MAP-2 immunolabelling confined to the cell bodies, dendrites and proximal axonal extensions by 5 DIV (Figure 3.1). At 6hrs PI, tau labelled neurites traversed the injury site. These sprouts, were not immunopositive for MAP-2, suggesting that they were predominately of axonal origin. High power magnification of growth cones labelled with antibodies to both tau and MAP-2 additionally confirmed that the growth cones under investigation were of axonal origin, ie tau immunopositive only (Figure 3.1E and F). Furthermore, the regenerative sprouts were immunopositive for βIII-tubulin, but showed little immunolabelling for the medium molecular weight neurofilament triplet protein (NF-M) (phosphorylation independent epitope). Similarly, labelled developing cultures revealed an abundance of βIII-tubulin throughout the neurites and extending into the growth cone with NF-M confined to the centre of the axon shaft (Figure 3.1C and D).

Comparison of growth cones on developing axons and on the tips of regenerative sprouts demonstrated distinct differences between the two structures. F-actin staining (Figure 3.2C and D), labelling with antibodies to the actin/membrane binding ERM proteins (Figure 3.2A and B) and DIC images (Figure 3.4) indicated that regenerating growth cones were notably smaller than their developmental counterparts, with shorter filopodial extensions. Immunolabelling with antibodies to βIII-tubulin, ERM proteins, tau and F-actin staining with phalloidin, demonstrated similar labelling patterns within the developing and regenerating growth cones. βIII-tubulin was confined to the central domain while ERM proteins were localised to the peripheral domain and the distal tips of filopodia (Figure 3.2A and B). Furthermore, there was colocalisation of F-actin and tau

throughout both developmental and regenerative growth cones (Figure 3.2C and D), with tau localised not only within the central domain, but additionally it crossed the transitional domain into the periphery of the growth cone.

3.3.2 There was a significant difference in axonal outgrowth between developing and regenerating cortical neurons *in vitro*

Analysis of growth cone position over a 2 hr period demonstrated that developing axons exhibit a wide range of movement involving rapid phases of extension and retraction with many changes in direction (Figure 3.3). This exploratory motility, was observed at 3, 5 and 7 DIV, however, the mean axonal outgrowth was shown to decrease with age of culture. Neurons at 3 DIV exhibited the greatest mean axonal extension over the imaging period (14.39 +/- 4.01µm n=5). At 5 DIV, the mean axonal extension was less (12.98 +/- 2,32µm n=5), but not significantly ($p>0.05$), with significantly ($p<0.05$) less axonal extension occurring at 7 DIV (6.19 +/- 0.98µm n=5). The post-injury sprouting axons also exhibited the exploratory motility observed in developing axons, however, their mean axonal extension was significantly ($p<0.05$) less at 6 hrs (4.03 +/- 0.89µm n=5) and 24 hrs post-injury (5.12 +/- 1.2µm n=5), in comparison to that observed in the developing axons at 3 and 5 DIV. There was no significant difference between the two PI time-points.

3.3.3 Growth cone morphology and behaviour in response to injury was significantly different in comparison to development

Time-lapse analysis of developing axons revealed a range of growth cone morphological forms. Furthermore, as previously reported, the morphology of the growth cone was related to the growth cones' behaviour (Skaliora et al., 2000). 'Extending' and 'retracting' growth cones exhibited simple streamline forms (Figure 3.4A). However, when a growth cone was 'pausing', with no net growth, the morphology dramatically changed to become large and considerably more complex in shape (Halloran and Kalil, 1994; Kalil, 1996; Skaliora et al., 2000). The same range of dynamic morphological alterations was not observed in the regenerating sprouts (Figure 3.4B). Instead growth cones of regenerating axons exhibited a constant morphology,

with less elaborate protrusions over the two-hour imaging sequence independent of behaviour. Dynamics after axonal injury was also investigated in 5 DIV developing neurons at 6 hrs PI, however, consistent with previous studies in our laboratory, no net growth or growth cone elaboration occurred (Figure 3.4C) (Dickson et al., 2000).

Growth cones of developing and regenerative axons underwent distinct and quantifiable phases of extension, retraction and pausing (Figure 3.5A). At 3 DIV, growth cones spent 46.39 +/-2.06% extending, 28.82 +/-1.30% retracting and 24.79 +/-1.31% pausing. At 5 DIV growth cones spent 40.09 +/-2.94% extending, 30.80 +/-1.67% retracting and 29.10 +/-3.20% pausing. At 7 DIV growth cones spent 38.55 +/-2.87 % extending, 26.67 +/-1.15% retracting and 35.19 +/-3.2% pausing. At 3 DIV, significantly ($p<0.05$) more extension and less pausing than 7 DIV was observed ($p<0.05$). The tips of post-injury regenerative sprouts behaved quite differently. At both 6 (57.75 +/-2.48% extending, 34.36 +/-2.56% retracting and 8.03 +/- 0.65 % pausing) and 24 hrs post-injury (55.56 +/-4.49% extending, 34.68 +/-2.04% retracting and 9.77 +/-1.53% pausing), the growth cones on the end of regenerative neurites spent a significantly ($p<0.05$) greater percentage of time extending and less time pausing. There was a 36% increase in extension behaviour and a striking 70% reduction in pausing behaviour in the growth cones of regenerative neurites compared to developmental growth cones.

3.3.4 Post-injury regenerative sprouts were unresponsive to growth factors

At 3 DIV application of both BDNF and GDNF significantly ($p<0.05$) increased the mean neurite length of neurons *in vitro* (Figure 3.5B). At 3 DIV the mean neurite length of the control treatment was 41.22 +/- 2.18µm (n=380). The mean neurite length with BDNF treatment was 54.86 +/-4.92µm (n=404); the mean neurite length with GDNF treatment was 53.97 +/- 3.36µm (n=445). Similarly at 5 DIV, both BDNF and GDNF treatment resulted in significant ($p<0.05$) increase in the mean neurite length. At 5 DIV, the mean neurite length of the control treatment was 64.56 +/-8.11µm (n=344), with BDNF treatment was 75.53 +/-9.15µm (n=329) and the mean neurite length with GDNF treatment was 79.71 +/-9.93µm (n=334). At both 3 and 5 DIV there was no significant ($p>0.05$) difference between the effects of the two growth factors, relative to

controls. BDNF and GDNF had little effect on neurite length at 7 DIV, the mean neurite length of the control treatment was 90.33 +/-7.23μm (n=349), with BDNF treatment was 93.34 +/-8.46μm (n=343) and the mean neurite length with GDNF treatment was 96.98 +/-9.22μm (n=313). Incubation of relatively mature cell cultures with BDNF, GDNF or a vehicle control following axonal bundle transection had no significant ($p>0.05$) effect on neurite outgrowth in the 16hrs following transection (Figure 3.5B). The mean neurite length of the control treatment was 59.03 +/-6.44μm (n=96). The mean neurite outgrowth with 20ng/mL BDNF treatment was 59.87 +/-5.27μm (n=112); the mean neurite outgrowth with 20ng/mL GDNF treatment was 57.22 +/-6.19μm (n=98). The punctate pattern of localisation of the BDNF receptor, Trk B, throughout the cell clusters and processes in development and relative maturity (Barnabé-Heider and Miller, 2003; Swanwick et al., 2004; Gomes et al., 2006) demonstrated widespread receptor expression by multiple cortical neuronal phenotypes, as shown by co-immunolabelling for βIII-tubulin (Figure 3.6).

3.4 DISCUSSION

Immunocytochemistry, with antibodies to both the axon and dendrite specific microtubule associated proteins, tau and MAP-2 respectively, enabled the visualisation of stages of neuronal morphogenesis - the rapid elongation of multiple dendrites and a single axon. Furthermore, the sprouting response of the mature injured axons *in* vitro (Dickson et al., 2000; Chuckowree and Vickers, 2003) was also demonstrated with axon-specific markers. Developmental axon extension requires the assembly and reorganisation of the cytoskeleton (Dent and Gertler, 2003), with the role of microtubules, neurofilaments and F-actin in this process well characterised (reviewed in Brandt, 1998). Recent data suggests that assumptions that the same dynamic processes are required for regenerative sprout elaboration may not be valid. Studies on regenerating lamprey reticulospinal axons indicate that regenerating axons appear to extend without growth cones or F-actin accumulation at their tips (Lurie et al., 1994; Zhang et al., 2005). It has been postulated that regenerative axonal outgrowth may be a direct result of protrusive forces created by tubulin and neurofilaments (Jones et al., 2006).

In the current study, DIC/live images, with F-actin staining and labelling for the ERM family of actin/membrane binding proteins demonstrated that the growth cones present on the tips of regenerative sprouts were smaller, with some lacking the complex morphology of developmental growth cones (Chuckowree and Vickers, 2003; Haas et al., 2004). Despite these differences, the regenerating and developing axons exhibited the same labelling profile for a range of cytoskeletal proteins. Both the developmental growth cones and the tips of the regenerating axons contained isoforms of microtubules, as indicated by tau and ßIII-tubulin labelling. Likewise, there was comparable localisation of F-actin and associated binding proteins, indicated by phalloidin staining and ERM labelling. However, immunoreactivity for the medium molecular weight neurofilament triplet protein, NF-M, consistent with previous investigations (King et al., 1997; Dickson et al., 2000; 2005), was confined to the neurite shafts proximal to the lesion, and was completely absent from regenerative and developmental growth cones.

This pattern of labelling has been reported for other intermediate filament proteins at these ages including α-internexin (Dickson et al., 2005) and the high molecular weight subunit of the neurofilament triplet proteins, NF-H (Dickson et al., 2000). The comparable expression and localisation of tau and ßIII-tubulin, NF-M and F-actin in post-injury and developing axons and growth cones suggests that regenerating axons possess the cytoskeletal capacity for motility and extension (Chuckowree and Vickers, 2003; Haas et al., 2004).

In agreement with *in vivo* studies, developing axons, *in vitro*, exhibited characteristic movement, such as outgrowth, retraction, stalling and turning and a mean decrease in outgrowth with age (Suter and Forscher, 2000; Gordon-Weeks, 2004). This decline in outgrowth, demonstrated *in vivo* (Li et al., 1995; Blackmore and Letourneau, 2006) may be representative of the decreased requirement for outgrowth as development progresses, and the axon reaches its correct target and synaptogenesis occurs. A significantly decreased rate of axonal outgrowth also occurred after injury, again indicating that maturing neurons undergo a decline in the expression of genes associated with developmental axonal growth (Cia et al., 1999), resulting in less regenerative capacity.

In vivo and *in vitro* analysis has shown that growth cone morphology correlates with characteristic movement (Halloran and Kalil, 1994; Kalil, 1996; Skaliora et al., 2000). In the current study, developmental growth cones spent a proportional percentage of time extending, pausing and retracting, which was highly comparable to characteristic behaviour analysis *in vivo* by neurons, at decision points in the developing brain (Skaliora et al., 2000). Regenerative growth cone behaviour was in striking contrast to that observed during development. The regenerating growth cones exhibited a greater tendency to extend and spent little time pausing. Growth cone pausing is implicitly linked to pathway selection (reviewed in Dent et al., 2003). Additionally, pausing of the primary growth cone of developing neurons has been highly correlated with interstitial axonal branching (Szebenvi et al., 1998; Kalil et al., 2000). The regenerating axons lack of pausing may result in impeded pathfinding and/or an inability to appropriately branch, both of which would contribute to a poor outcome post-injury. Furthermore,

aberrant sprouting has been documented in both *in vitro* (McKinney et al., 1997) and *in vivo* (Salin et al., 1995; Kerschenstiner et al., 2005) studies of CNS trauma. This sprouting may be attributable to an intrinsic lack of the regenerative growth cones ability to 'path-find' appropriately.

The inability of mature axons to regenerate may also be due in part to the absence of growth permissive signals or the presence of growth inhibitory cues at the site of injury. The current chapter investigated the application of two known growth cues, in an environment absent of inhibitory cues. Previous studies by Goldberg and colleagues demonstrated BDNF, GDNF and ciliary neurotrophic factor, to be the most potent axon growth factors on retinal ganglion cells (Goldberg et al., 2002). Interestingly, both BDNF and GDNF act on the same family of receptors and potentially affect the same downstream signalling pathway for neurite outgrowth. Acting on tyrosine kinases receptors (BDNF acting on the Trk B receptor and GDNF eliciting its effect on neurons through a multicomponent receptor complex involving GFRα-1 and Ret) both BDNF and GDNF have been implicated in survival, differentiation and neurite outgrowth (reviewed in Korsching, 1993). In this chapter's experiments, both growth factors caused an increase in neurite extension during development (Tucker et al., 2001; Airaksinen and Saarma, 2002; Goldberg et al., 2002), with the effect decreasing with age (Niwa et al., 2002). The increase in neurite length was irrespective of the growth factor applied, indicating the degree of functional redundancy in the signalling pathways. In contrast, neither growth factors affected post-injury axonal outgrowth, suggesting that growth promoting signals alone is not sufficient to overcome the intrinsic inhibition of mature axons to regenerate. Growth factors have been shown to promote terminal axonal sprouting in the mature nervous system in other neuronal phenotypes including cortical neurons (Zhou and Shine, 2003; Hafidi et al., 2004). Differential expression of BDNF and GDNF receptor components has been demonstrated in neurons of the cortex in the immediate post-injury period (Bakshi et al., 2006), however, the present experiments demonstrated robust and consistent Trk B localisation in the cortical cultures at both 5 and 21DIV across multiple neuronal phenotypes, therefore making it unlikely that lack of responsiveness was due to simply to lack of receptor expression. This data further supports recent reports indicating the

presence of Trk B receptors in cultured cortical neurons, both excitatory and inhibitory, from 1 to 16DIV (Gomes et al., 2006) and in cultured hippocampal neurons from 1 to 21DIV (Swanwick et al., 2004).

Classically, the local sprouting of axons at the site of injury has been taken to indicate that CNS neurons successfully initiate axon regeneration that is then impeded by the non-permissive environment. Whereas recent advances have lead to attempts at identifying and overcoming the extrinsic inhibitory factors that exist in the CNS environment the mechanisms underpinning the intrinsic incapacity of mature CNS neurons to recover from trauma remain to be fully elucidated. The current Chapter provides significant insight into why regenerative attempts are limited or aberrant, with increased understanding of this phenomena essential to the development of post-trauma interventions. Our results indicating a preserved cytoskeletal capacity to respond to trauma suggest that the differences responsible for the variation may lie at the level of the growth cone, specifically with regards to expression of growth and guidance factor receptors.

4 AXONAL SHEARING IN MATURE CORTICAL NEURONS INDUCES ATTEMPTED REGENERATION AND THE REESTABLISHMENT OF NEURITE POLARITY

4.1 INTRODUCTION

There are varying degrees and regions of focal and diffuse injury that can occur in TBI (reviewed in Farkas and Povlishock, 2007), which plays a role in making the pathobiology complex and intervention difficult. A full understanding of the response of the brain to various modes of injuries and mechanisms underlying these responses is a fundamental priority in neuroscience research. Thus, a range of animal models both *in vitro* and in vivo has been developed in an endeavour to elucidate the response of the brain to TBI. *In vitro* animal models, while largely two dimensional, can be advantageous in unravelling specific pathology and mechanisms as they allow for the precise control of the extracellular environment, are repeatable, have relatively low economic costs (Morrison et al., 1998) and have been shown to produce results consistent with those obtained from comparable *in vivo* models (Dotti et al., 1988; Banker and Goslin, 1988; King et al., 2001; Dickson et al., 2005; Chuckowree and Vickers, 2003).

As previously discussed, successful regeneration will require the survival and appropriate polarisation of injured neurons with the formation, robust extension and pathfinding of the axonal growth cone. Furthermore, the neuronal response to injury is heavily influenced by the action of surrounding non-neuronal cells such as the oligodendrocytes, astrocytes and microglia (Aldskogius and Kozlova 1998; Tao and Aldskogius 1999; Cullheim and Thams, 2007; Carlson et al 2009). Astrocytes undergo adaptive reactive alterations in response to injury involving proliferation, hypertrophy and the secretion of various factors that culminate in the formation of the glial scar (Maxwell et al., 1990; Raivich et al., 1999; Chen et al., 2003 a,b and Galtry et al., 2007). Thus, the response of mature neurons to injury is dependent upon the neuronal injury and the surrounding cells. Firstly, the intrinsic response of axons to injury remains to be fully elucidated. Secondly, how the inhibitory environment affects the

response of injured axons still needs to be clarified. For example while astrocytes facilitate brain function in an uninjured brain, whether astrocytes play an overall beneficial or adverse function after brain injury remains unclear.

While axons are particularly vulnerable in TBI (Maxwell et al., 1997), focal injury of a specific single axon rarely occurs (Farkas and Povlishock, 2007). Rather, most axonal injury occurs in a diffuse manner affecting multiple neuronal and non-neuronal cells (Stone et al., 2004; Singleton and Povlishock, 2004; Gallyas et al., 2006). Thus the current chapter endeavoured to develop an *in vitro* model of axonal injury that involved a total shearing of many axonal process from the neuronal cell body. The ability of relatively mature neurons to survive and attempt a regenerative response after this novel model of axonal shearing was under investigation. The axonal shearing injury was performed through enzymatic dissociation and mechanical axonal stripping which resulted in a complete stripping of axons from the cell body clusters. The response of the neurons to this mode of axonal injury, and in comparison to the transection model of injury previously described in Chapter 1, was investigated using a combination of live imaging and immunocytochemical analysis. Furthermore, the effects an activated astrocyte monolayer had on the response of the regenerating axons was also determined.

4.2 MATERIALS AND METHODS

4.2.1 *In vitro* model of structural injury

Primary neuronal culture was prepared as described in Chapter 2.1. Structural injury, using a combined chemical and mechanical approach, was performed on cortical neurons in long-term culture (21 DIV). Specifically, cultures were treated with 200µL of 0.05mg/mL trypsin/EDTA (Sigma, USA) for 5mins at 37°C, at which time neuronal cells clusters were beginning to loosen and lift from the underlying substrate. Neuron clusters were then mechanically dislodged from the coverslip using a cell scraper (Iwaki, Japan). 200µL of initial Neurobasal™ media was added to each well to inhibit trypsin activity prior to centrifugation (200g, 5 mins). The cell pellet was resuspended in initial Neurobasal™ media and gently triturated through a 1mL pipette. This did not result in the formation of a single cell suspension, but instead maintained the structural integrity of the original cell clusters, and the processes within it, whilst completely removing the interconnecting axonal bundles. Suspended cell clusters were re-plated on poly-L-lysine coated coverslips in 1mL of Neurobasal™ initial plating media (see above). At 24 hrs post-injury, media was removed and replaced with 2ml of Neurobasal™ supplemented with 2% B27, 0.5mM L-glutamine and penicillin/streptomycin antibiotics. To investigate the interaction between injured neurons with astrocytes, structurally injured neurons were also re-plated onto a confluent astrocyte monolayer. As a control for astrocyte reactivity, supernatant from the injured neurons was also plated onto an astrocyte monolayer. For cell proliferation studies 5-bromo-2'-deoxyuridine (BrdU, Sigma, USA, 5mM) was applied to cultures at 4 hrs post-injury and the cells fixed at 24 hrs post-injury.

4.2.2 Immunocytochemistry

Cultures were fixed in 4% PFA/0.01M PBS for 30mins at room temperature on an orbital shaker at either 3 or 21 DIV and 6 or 24 hrs post-injury and double-immunolabelled with a range of primary antibodies (as detailed in Table 2.1), as previously described (Chapter 2.4). To label for filamentous actin (F-actin), cultures were incubated with AlexaFluor® 594 phalloidin (dilution 1:200; Invitrogen, USA), for

30mins after immunocytochemistry. Immunolabelling was visualised using microscopes and cameras detailed in Chapter 2.4.

4.2.3 Live Imaging

Time-lapse imaging was conducted as previously described (Chapter 2.4; Chapter 3.2.4).

4.2.4 Statistical analysis

For quantitative analysis of mean fluorescence intensity, images were captured at the same exposure, time and magnification (400x). Regenerative sprout lengths and percentages with growth cones (n= 473) and growth cone area (n=400 for each condition) were quantitated with ImageJ software. ANOVA, with *post hoc* comparisons (Bonferroni test), and Students' t-test was performed on quantitative data as appropriate using Prism v4.0 software (Graphpad, USA).

4.3 RESULTS

4.3.1 Cortical neurons survive injury and exhibit a post-injury regenerative response

Dissociated cortical neurons adhered, formed cell clusters and elaborated neurites within the first week *in vitro*. By 21 DIV neuronal clusters, interconnected by large networks of axonal bundles, were extensive across the coverslip surface (Dickson et al., 2000; Chuckowree et al., 2003; Haas et al., 2004) (Figure 4.1A,B).

Neuronal clusters were completely stripped of extending axonal processes through a chemical - trypsin mediated, dissociation from the substrate combined with mechanical removal of the clusters using a cell scraper, and the remaining axonal connections through trituration. By 6hrs post-injury, neurons that had been replated onto a new substrate of poly-L-lysine, remained clustered, however, no neurites extending from the clusters were present (Figure 4.1C). By 24hrs post-injury the injured neurons had elaborated new processes, extending out on the substrate, mean length 67.31 +/- 15.01μm (Figure 4.1D). Unlike the tightly associated axon bundles present prior to injury, the newly sprouting neurites were present as single processes (Figure 4.1E) that by 7 DIV were able to connect with other surrounding cell clusters (Figure 4.1F).

Previous investigations indicate that cultured neurons derived from the cortex represent a heterogeneous population of cells (Dickson et al., 2000; Chuckowree et al., 2003; Haas et al., 2004). To determine if the post-injury sprouting response was restricted to particular subsets of this population, injured neurons were fixed at 24 hrs post-injury and immunolabelled for cell-type specific proteins. A population of the surviving injured neurons that elaborated sprouts post-injury were positive for calretinin (Figure 4.2), a calcium binding protein localised to a subset of GABAergic interneurons (Pappas and Parnavelas, 1998). However these sprouts remained locally within the neuronal cell cluster and did not extend out onto the poly-L-lysine substrate, beyond 0.00μm from the cell cluster border. Other sprouts emanating from injured neurons

were immunopositive for SMI312, an antibody that labels phosphorylated neurofilament triplet proteins (Figure 4.2). SMI312 sprouts were emanating out from the cluster into the lysine substrate. No colocalisation between labelling for calretinin and SMI312 was evident.

4.3.2 Neurite lesion does not stimulate neurogenesis

BrdU incorporation with double labelling immunofluorescence studies were performed to determine whether neurogenesis had occurred in our *in vitro* neurite lesion model. The injured neurons that had adhered to the fresh substrate did not incorporate BrdU (Figure 4.3A-C). Double labelling with nestin an intermediate filament protein expressed by neural precursors (Figure 4.3D) (for a review see Michalczyk and Ziman, 2005) and the neuronal marker tau (Figure 4.3E), indicated that there were nestin positive cells within the cell cluster, however these cells did not co-localise with tau (Figure 4.3F). Nestin positive cells (Figure 4.3G) were also immunopositive for the antibody directed towards the astrocyte marker protein glial fibrillary acidic protein, GFAP (Figure 4.3H,I).

4.3.3 Relatively mature neurons demonstrate neurite polarisation following injury

Chapter 3 demonstrated that at 21 DIV, prior to structural injury, cultured neurons expressed specific cytoskeletal associated proteins indicative of cellular maturity such as tau and MAP-2 (Chuckowree and Vickers, 2003; Haas et al., 2004). In the current investigation Tau was localised throughout cell bodies and axonal bundles (Figure 4.4A). MAP-2, a somato-dendritic marker was confined in its expression and specifically labelled the cell body cluster and dendrites extending only a short distance from the cluster (Figure 4.4B), but was not present within interconnecting process bundles (Figure 4.4C). This polarisation of neuronal processes has been previously established to occur, *in vitro*, during early development (Dotti et al., 1988; Banker and Goslin, 1988), with the elaboration and specification of an axon occurring at 48hrs (de Anda et al., 2005). At 24hrs post-injury, confocal microscopy revealed that the processes emanating from the cell cluster were already immunopositive for tau only

(Figure 4.4D and G) with MAP-2 expression again restricted to cell bodies and minor processes confined to the cell cluster (Figure 4.4E and H). MAP-2 and tau expression was compartmentalised with no-colocalisation of expression (Figure 4.4F and I). Additionally, tau labelling indicated that the morphology of the sprouts emanating from the cell clusters was consistent with that reported for axons during development (Dotti et al., 1988).

4.3.4 Regenerating growth cones are smaller than developmental growth cones but are cytoskeletally comparable

By 24 hrs post-injury, approximately half of the regenerative axonal sprouts (57.3 +/- 3.19%) were tipped by growth cone like structures. Comparative analysis of the mean area of the growth cones at the end of developing and post-injury axons revealed that the structures on the tips of regenerative neurites were significantly ($p<0.05$) smaller than their developmental counterparts at 3 DIV (Figure 4.5A). Replating the injured neurons onto the astrocyte monolayer resulted in increased GFAP expression and altered astrocyte morphology, indicative of increased reactivity (Raivich et al., 1999) (Figure 4.5B,C). However, comparison between the mean areas of growth cones of neurons plated onto a substrate of poly-L-lysine or an astrocyte monolayer revealed no significant ($p<0.05$) differences in growth cones area in both developing neurons and injured neurons (Figure 4.5A). Application of either uninjured embryonic neurons or conditioned media from injured neurons did not elicit astrocyte reactivity (data not shown).

Immunocytochemical labelling with a range of cytoskeletal and growth associated proteins in the growth cones of developing axons and on the tips of the axonal sprouts further demonstrated this distinct difference in size however, specific protein localisation patterns were comparable (Figure 4.6A-L). Growth cones on the tips of developing axons possessed complex tau (Figure 4.6A) and F-actin (Figure 4.6D) labelled structures. Tau and F-actin expression was concentrated in the central domain of the developmental growth cone and also crossed into the transitional domain into the periphery of the growth cone (Figure 4.6C and F). The growth cones on the tips of regenerative sprouts had a less extensive pattern of tau (Figure 4.6G) and F-actin

(Figure 4.6J) localisation that was evident only in the central domain of the growth cone (Figure 4.6I and L). These smaller regenerative growth cones appeared to be lacking the peripheral domain evident in the developmental growth cones. Alpha-acetylated tubulin was localised predominately to the neurite shaft in both developmental (Figure 4.6B,C) and regenerative (Figure 4.6H,I) growth cones and GAP-43 expression was present predominately within the central domain of both developing (Figure 4.6E,F) and regenerating growth cones (Figure 4.6K,L).

4.3.5 Growth cone dynamics of regenerative sprouts differ from development

Live imaging over a two-hour period was used to investigate the dynamics of the growth cones of sheared regenerative axonal sprouts compared to that occurring during development (Figure 4.7A,B). Analysis of growth cone position over this time demonstrated that developing axons exhibit a wide range of movement involving rapid phases of extension and retraction with many changes in direction, as previously reported in Chapter 3 (Figure 4.7A,B). Analysis of regenerative growth cones, post-injury, revealed that the growth cones underwent distinct and quantifiable phases of extension, retraction and pausing. The growth cones present on the tips of regenerative sprouts, at 24hrs post-injury, spent 55.78 +/- 1.61 % extending, 33.93 +/- 1.27 % retracting and 10.28 +/- 0.61 % pausing (n=5) (Figure 4.7B). This data indicates that growth cones post-injury spent a significantly ($p<0.05$) greater percentage of time extending and a significantly ($p<0.05$) lower percentage of time pausing, as compared to developmental growth cones at 3, 5 and 7 DIV; replicating the data for regenerating growth cone behaviour following axonal transection, investigated in Chapter 3.

4.4 DISCUSSION

TBI can be categorised into two broad phases; the direct injury occurring from the tearing of shearing of axons at time of impact and the sequence of events occurring after injury, known as secondary injury. The current experiments utilises a simple, reproducible and novel *in vitro* model replicating the primary axonal tearing and subsequent regenerative sprouting in cultured primary cortical neurons. This model utilises mechanical tearing or shearing of axons as an injury model, which may be more representative, than the discreet transection model, of the widespread neuronal and non-neuronal injury occurring *in vivo.* To validate this model and further define the post-injury response the dynamic and cytoskeletal axonal changes occurring after the injury was investigated and found a stereotype reminiscent of those observed during experimental and *in vivo* TBI and distinct from initial developmental outgrowth.

While an increase in neurogenesis has been reported after brain injury *in vivo* (for example: Braun et al., 2002; Emsley et al 2005), whether such an injury evokes a neurogenic response that can contribute to repair remains contentious (Holmin et al., 1997; Salman et al. 2004). Neurogenesis as a result of injury was not observed in the current *in vitro* model, as indicated by an absence in BrdU uptake and nestin labelling. Thus the neuronal response observed in this model can be attributed to the resident population of relatively mature cortical neurons.

In this chapter neuronal cultures were immunolabelled for the protein calretinin, a cell type specific marker that labels a subset of GABAergic interneurons (Pappas and Parnavelas, 1998). Calretinin immunopositive neurons survived the injury and extended processes from the cell clusters after replating. Similarly, another subset of axonal sprouts were labelled by the antibody SMI312, which specifically recognises phosphorylated neurofilament triplet proteins. SMI312 has been previously reported to be expressed in pyramidal neurons, albeit not exclusively (Masliah et al., 1993; Dickson et al., 2000; 2005). These results indicate that there was not one specific cell type that survived the injury, instead suggesting comparable potential to survive and attempt regeneration across multiple neuronal populations. However, only the SMI312

immunolabelled pyramidal neurons attempted to regenerate in the form of extending sprouts out from the cell cluster, with no calretinin immunolabelling present away from the cell cluster, suggesting that the regenerative sprout response observed in the Chapter 3 and the current Chapter, may be neuronal cell type specific.

As previously discussed in Chapter 3, the ability of neurons to polarise and elaborate a single molecularly and morphologically defined axon, tipped by a growth cone, and multiple, equally specific dendrites is critical to neuronal function and the establishment of the precise and extensive networks that characterise the brain. Cortical neurons, *in vitro*, display markers indicative of neurite polarisation by 3 DIV (Dotti et al., 1988). Previous investigations in Chapter 3 established that the tightly associated neurite bundles emanating from the neuronal cell clusters, derived from E18 rats and grown for 21 DIV, are primarily composed of axons, as evidenced by labelling for the axon specific MAP tau and no immunoreactivity for the dendritic marker MAP-2. In the present set of experiments, these processes were removed, whilst leaving the cell cluster, and associated synaptic connections within the cluster, intact. As such, the model is one of partial deafferentation, comparable to *in vivo* injury (reviewed in Chuckowree et al., 2004). At 24 hrs post-injury, morphologically axon-like structures, had emanated from the cell body cluster. Labelling for the dendrite specific protein MAP-2 indicated that dendritic processes remained confined to the networked cell clusters, consistent with that reported prior to injury. Furthermore immunocytochemically, after injury the differentiation of single axons and multiple dendrites, demonstrated by distinct compartmentalised tau and MAP-2 labelling (Bernhardt and Matus, 1984; Binder et al., 1985). However, regenerative axonal sprouts that formed post-injury did not fasciculate and extend together, as seen in the initial development of the culture. Instead single axonal sprouts were distributed non-uniformly around the cell cluster.

The axon-like structures, post-injury, were tipped by a characteristic growth cone. Previous investigations, including those in Chapter 3, have suggested that growth cones on the tips of regenerating cortical neurons *in vitro* posses a different morphology to the growth cones present in development (Chuckowree and Vickers, 2003; Haas et al.,

2004). Analysis of the mean growth cone area for developing and regenerating growth cones indicated that indeed, regenerating growth cones in the current model are significantly smaller than their developmental counterparts. Interestingly, there was no significant change in mean growth cone area for growth cones in contact with reactive astrocytes. Classically, astrogliosis has been considered one of the fundamental extrinsic limitations to regeneration (Silver and Miller, 2004; Fawcett 2006). However, reactive astrocytes are thought to have both beneficial and undesirable effects. Moreover, *in vitro,* many studies have indicated that reactive astrocytes can be growth permissive (Chung et al., 2004). The astrocyte monolayer became reactive when exposed to the injured neurons, classified by an increase in GFAP expression and altered morphology (Raivich et al., 1999). The absence of reaction from both conditioned media and uninjured immature neurons indicated that this reactivity may be mediated by direct cell contact of the injured neurons. There was no significant change in mean growth cone area for growth cones in contact with reactive astrocytes compared to poly-L lysine substrate. The smaller size of the growth cones in regeneration compared to development, coupled with lack of response to reactive astrocytes, is further evidence that these structures have a diminished capacity to respond to the extracellular environment.

Despite, these distinct morphological size differences the regenerative growth cones exhibited a similar complement and expression pattern of cytoskeletal proteins compared to that of their developmental counterparts (for review see Suter and Forscher, 2000; Gordon-Weeks, 2004; Pak et al., 2008). This included the presence of tau (Black et al., 1986), GAP43 (Goslin et al., 1988; 1990) and an F-actin network (Bradke and Dotti, 1999). The growth cone is vital for the translation of extracellular cues into pathway selection. This guidance is enabled through the interaction between various components of the cytoskeleton that are expressed throughout the specific domains of the growth cone; the central domain the transition zone and the peripheral domain. In the central domain no actin superstructures are present, however this area is rich in microtubules. The peripheral domain contains two distinct superstructures based on actin filaments, the linear actin bundles comprise filopodia which protrude outward and mesh-like actin gels comprise lamellipodia, located between the filopodia (For

recent review see Pak et al., 2008). Hence the diminished surface area of regenerating growth cones, and the lack of an extensive peripheral domain may offer an explanation for the diminished capacity to 'pause' that was evident in regenerative axonal sprouts – thus morphologically resembling the dystrophic growth cones characteristic of spinal cord injury *in vivo* (Kwon et al., 2002) and in *in vitro* models that have also proven to be highly dynamic but aberrant in their ability to forward progress (Tom et al., 2004).

In agreement to Chapter 3, developmental growth cones spent a proportional percentage of time extending, pausing and retracting, which was highly comparable to characteristic behaviour analysis *in vivo,* by neurons at decision points in the developing brain (Skaliora et al., 2000). As stated, growth cone pausing is implicitly linked to pathway selection, and occurs *in vivo* in development when a growth cones reaches a decision region. Regenerative growth cone behaviour was in striking contrast to that observed during development. The striking lack of the ability to pause, demonstrated by the regenerating growth cones in comparison to development, in coherence with Chapter 3, further suggests a lack of ability to path-find of regenerating growth cones. Additionally the growth cone dynamics were highly comparable to Chapter 3, indicating that regenerating characteristics are transferable over a range of injury models.

5 FOCAL DAMAGE TO THE ADULT RAT NEOCORTEX INDUCES WOUND HEALING ACCOMPANIED BY AXONAL SPROUTING AND DENDRITIC STRUCTURAL PLASTICITY

5.1 INTRODUCTION

Ultimately, an appropriate adaptive brain response to trauma will require the induction of neurogenesis and either directed regeneration or compensatory plasticity of neural pathways. Structural injury to the brain does evoke a distinct sequence of events indicative of an attempt to repair and heal, including the activation of microglial, oligodendroglial precursor, meningeal, astrocyte and stem cell populations (Reviewed in Fawcett and Asher, 1999). Alterations in neural progenitor populations and cell proliferation have been demonstrated in diverse models of experimental brain lesion and stroke conditions and are proposed to contribute to post-lesion brain recovery (Clarke et al., 1994; Duggul et al., 1997; Holmin et al., 1997; Kernie et al., 2001; Arvidsson et al., 2002; Chen et al., 2003b; Douen et al., 2004; Salman et al., 2004). However, whether brain injury evokes a neurogenic response that contributes to functional recovery currently remains contentious, with the presence of neurogenic events differing for different injury paradigms (Holmin et al., 1997; Kernie et al., 2001; Kuroda et al., 2002; Rice et al., 2003; Salman et al., 2004; Yu et al., 2008). It is now established in Chapter 3 and 4, in agreement with previous studies, that neurons can also actively react to injury through the formation of reactive axonal sprouts. However, another mode in which neurons respond actively to injury is through the altered connectivity of pre-existing pathways, compensatory plasticity (reviewed in Chuckowree et al., 2004; Carmichael, 2006; Fitzgerald and Fawcett 2007; Macias, 2008).

Examples of neuronal plasticity, manifesting as alterations in synaptic strength and wiring, remodelling of axonal and dendritic arbors and changes in dendritic spine and axonal bouton turnover, have been observed in the mature brain and in response to alterations in sensory experience and injury/lesion (for a selection of recent examples see Grutzendler et al., 2002; Knott et al., 2002; Trachtenberg et al., 2002; Majewska and

Sur, 2003; Portera-Cailliau et al., 2003; Holtmaat et al., 2005; Tailby et al., 2005; Lee et al., 2006; Majewska et al., 2006; Brown et al., 2007; Brown et al., 2008; Lee et al., 2008; Yamahachi et al., 2009; Brown et al., 2010). However, the specificity and degree of remodeling that can occur in response to injury has not been fully elucidated. Cortical neurons demonstrate axonal and synaptic remodelling in response to various models of injury (for example King et al., 2001; Knott et al., 2002; Trachtenberg et al., 2002; Majewska et al., 2006). Reorganization such as this may correlate with functional recovery not only in stroke (Carmichael, 2003; Carmichael, 2006; Brown, 2008; De Filippo et al., 2008), but comparable plasticity may also account for the recovery observed after forms of spinal cord injury (reviewed in Edgerton, 2004; Dunlop, 2008). Recent research indicates that populations of interneurons within the barrel cortex demonstrate adaptive plasticity, specifically with regards to the remodelling of dendritic arbors (Lee et al., 2006; Lee, 2008). Modifications of the dendritic tree and synaptic contacts may also occur following injury, with studies showing that the plastic potential of cortical dendrites are enhanced following injury (Kolb and Gibb, 1991; Jones and Schallert, 1992), lasting over two weeks post-injury (Jones and Schallert, 1992). Interestingly, studies have indicated that not all subpopulations of neurons are equal in their capacity for such adaptive structural plasticity. For example, interneurons in the intact neocortex have been demonstrated to be capable of dendritic arbor remodelling while pyramidal neuronal subpopulations remain stable (Lee et al., 2006).

The current investigations utilised a model of focal brain injury to generate a discrete unilateral lesion in the adult rodent somatosensory cortex. The cellular response to injury was investigated over a time course of up to 14 DPI (days post-injury) utilising immunohistochemistry and transmission electron microscopy (TEM). To determine the regenerative and plastic potential of different subpopulations of neurons following focal *in vivo* injury, the sprouting response and morphological plasticity of resident interneuron and pyramidal neuron populations around the injury site was investigated utilising immunohistochemistry, confocal and multi-photon microscopy.

5.2 MATERIALS AND METHODS

5.2.1 *In vivo* brain injury

Acute focal neocortical injuries were performed on adult male Hooded-Wistar rats (250-270g, corresponding to approximately 8 weeks old) and adult male thy1 GFP-M mice (between 8-10 weeks old), the injury protocol described in Chapter 2.3. The thy1 GFP-M mice were kept as a homozygous mouse colony, hence no routine genotyping was required. Thy1 GFP-M mice have a limited expression in a variety of neurons, including neurons within the cortex, cerebellum, motor neurons within the spinal cord, retinal ganglion cells and dorsal root ganglia (Feng et al., 2000). Importantly, GFP-M mice were utilised by the current studies due to the fact that the expression pattern within the neocortex was restricted to a subpopulation of pyramidal neurons (Feng et al., 2000). Following recovery, animals were terminally anaesthetised (pentabarbitone sodium, 140mg/kg) and transcardially perfused with 4% PFA/0.01M PBS, following a 0.01M PBS clearing procedure, at a range of post-injury time intervals up to 14 days following injury ($n \geq 5$ animals per time-point at 1, 7 and 14 DPI) and brains were processed for either immunohistochemistry or transmission election microscopy (TEM) as described in the relevant sections below. Non-injured, age-matched controls corresponding to the 1, 7 and 14 DPI time-points (n=5 animals per time-point) were also perfused

To determine the phenotype of cells proliferating within the first 7 days following injury, animals were administered with 12.5mg/ml solution of the BrdU, (Sigma, USA) in 7mM NaOH/0.9% NaCl by intraperitoneal injection. Animals were injected with BrdU (25mg/kg/day), between 1 and 6 DPI and were transcardially perfused (4% PFA/0.01M PBS at 7 days following injury. Prior to immunohistochemistry, antigen retrieval was performed by incubating sections in 2M HCl for one hour at 37°C, followed by three neutralisation washes in 0.1M borate buffer (pH 8.5).

5.2.2 Immunohistochemistry

Brain sections (50μm vibratome) were collected coronally and also horizontal to the pial surface, through the injury site, from control and experimental animals and were

immunohistochemically labelled in single as well as double-labelling combinations, with a range of cell specific antibodies (Table 2.1), as described in Chapter 2.4.

5.2.3 Ultrastructural/TEM studies

Animals were injured, as above, and then perfused at 7 or 14 DPI with 4% PFA/2%glutaraldehyde/0.01M PBS. Brains were post-fixed in the same fixative solution overnight at 4°C and then stored in PBS until sectioned. Small regions of the injury site (or equivalent regions of control non-injured brains) were removed and trimmed to 1-2mm cubes. Tissue blocks were then osmicated, stained with uranyl acetate, dehydrated and embedded in Epon resin. Thick (1μm) plastic survey sections were cut and stained with 1% toluidine blue in 1% borax until a suitable region was found. Thin sections (70-90nm) were then cut on a Reichert Ultracut ultramicrotome and placed onto copper-palladium mesh grids. Following staining with uranyl acetate and lead citrate, grids were examined using a Philips CM100 transmission electron microscope.

5.2.4 Analysis of dendrite orientation

For these investigations five male rats and five male thy1 GFP mice at 14 DPI were matched to five-control animals, for both species, with focal injuries in the somatosensory cortex, as detailed above, sham injury performed post-mortem. Vibratome brain sections (80μm) were serially sectioned through the injury site for each control and experimental animal, and for rat sections, immunolabelled with calretinin, the calcium binding protein localised to a subset of GABAergic neurons (Pappas and Parnavelas, 1998). A series of digital images were captured at optimal z-axis intervals (maximum 2.5μm), using a Zeiss LSM 510 multi-photon/confocal dual purpose microscope with Zen software. Multi-photon microscopy was utilised to enable visualisation through the whole brain sections. Four 20x fields of view were captured around each injury site for two separate sections from each animal and all cells within the field of view identified for analysis. Flattened 3D projection stacks were overlaid

with a target that had an 80μm circumference (for example see Figure 5.6 A and B), and analysed blinded to experimental conditions, using ImageJ© freeware.

To quantify the orientation of the dendrites, the angle of all dendrites intersecting the 80μm circumference for each neuron was measured, relative to the direction of the injury site. This was calculated by drawing a line from the injury site to the centre of each soma and then measuring the acute angle between the intersection points for each dendrite at the 80μm circumference. Thus for each dendrite this angle of intersection ranged between 0 and 90°. Therefore an angle of 0° indicates that the dendrite is orientated directly in line with the injury site (either towards or away from) and angle of 90° indicates that the dendrite is orientated laterally relative to the injury site, with a random distribution having an expected angle of 45° (Figure 5.6A). From this data the mean dendrite angle per neuron (θ) was calculated. Thus the null hypothesis was that there was no significant change in the mean dendrite angle, relative to the injury site, at 14 DPI in comparison to sham-injured control. Mean dendrite angle in correlation with distance from the injury site were investigated for significance using two-way ANOVA, t-tests, and linear regression, respectively with the InStat statistical package (GraphPad). Circular variance of angle distribution was analysed with the Oriana 3.0 statistical package.

A limitation to analysing changes in mean dendrite angle, is that the statistical analysis can demonstrate significant changes in orientation relative to the control, but cannot give an indication of the direction of the alteration. To determine the direction of any significant changes in dendrite orientation, the polarity of all dendrites was determined for calretinin positive neurons. The number of dendrites in four quadrants - proximal, lateral 1 and 2 and distal (Figure 5.7A), to the injury site, intersecting at 80μm from the cell body, was calculated to determine dendrite polarity, and analysed using a two-way ANOVA (InStat, GraphPad).

5.2.5 Confocal and multi-photon microscopy

All confocal and multi-photon images collected in this study were obtained on a Zeiss LSM 510 microscope, as described in Chapter 2.3. Both lasers were passed through either a 20x/0.8 NA plan-apochromat (Zeiss) objective to visualise the entire injury site and a 64x/0.8 NA plan-apochromat (Zeiss) oil immersion objective for high power images. For quantification of potential dendritic changes, calretinin immunolabelled cells were visualised with the HeLa 594 laser passing through a 20x/0.8 NA plan-apochromat (Zeiss), the laser and pinhole settings remaining constant. GFP cells were visualised with a Mai Tai multiphoton laser, at the wavelength 850 nm, with constant power, passing through a 20x/1.0 DIC VIS IR W Plan-apochromat (Zeiss) water immersion objective.

5.3 RESULTS

5.3.1 Focal injury induces an active healing response culminating in the formation of a glial scar and neovascularisation

The cellular alterations following localised injury to the neocortex were examined at 1, 7 and 14 DPI. Analysis of cortical post-injury material sectioned in the horizontal plane revealed distinct cellular changes as the response to injury progressed, within this tissue core and surrounding the injury site, that were less evident using traditional coronal sectioning. Focal neocortical injury resulted in substantial tissue destruction at the site of the lesion and subsequent alterations indicative of attempted brain repair. Microscopic analysis demonstrated no detectable histopathological changes in control brains or in brain regions contralateral to the lesion site, evidenced with both resin sections (Figure 5.1A and F) and also with immunolabelling for GFAP (Figure 5.1K). The neuropil in control non-injured resin sections was essentially homogenous, tightly associated and dense (Figure 5.1A and F). At 1 DPI, tissue destruction was noted within the injury site, with a clear needle tract being visible (Figure 5.1B). This lesion site at 1 DPI was characterised by massive red blood cell infiltration (Figure 5.1G). By 7 DPI the injury site had increased in size and now ranged from 200 – 300μm in diameter. The blood cells that were present at 1 DPI had cleared and neovascularisation of the lesion site had commenced with the area infiltrated by occasional new blood vessels and sparsely populated with cells and processes (Figure 5.1C). The neuropil within the injury site, however, remained significantly less dense than surrounding tissue (Figure 5.1H). Immunolabelling for GFAP demonstrated that the injury and peri-lesion site had now become filled with reactive astrocytes with their processes directed towards the central lesion site (Figure 5.1M). At 14 DPI, blood vessels permeated the injury tract (Figure 5.1D and I, arrows) and a significant proportion of the tract had closed together leaving a central dense GFAP labelled core (Figure 5.1N) (King et al., 2001), with a glial scar extending approximately 500μm from the edges of the lesion (Figure 5.1N and O). GFAP immunolabelling was greatest directly adjacent to the injury site, as indicated by increased immunoreactivity, with this labelling progressively decreasing with increasing distance from the injury (Figure 5.1N and O).

Ultrastructural studies of the central lesion site at 7 and 14 DPI demonstrated, in support of immunohistochemical analysis, the presence of activated microglia with inclusions (Figure 5.1E) and also proliferating cells (Figure 5.1J).

5.3.2 Cortical injury induces neural progenitor proliferation but not neurogenesis

As noted above, proliferating cells were a prominent feature of the tissue surrounding the injured neocortex (Figure 5.1J). These cells were further characterised utilising the progenitor marker nestin and the mitotic markers, BrdU and PCNA. Nestin immunoreactive cells were not evident in the grey matter of the neocortex of control non-injured brains (Figure 5.2A), although some labelling was observed in the periventricular region (data not shown). By 7 DPI, nestin immunoreactive profiles were localised throughout the neocortical laminae and were especially abundant adjacent to the injury site (Figure 5.2B and C). Alterations in the localisation of nestin immunoreactivity within the neocortex were restricted to the ipsilateral cortex and were not observed in cortical tissue contralateral to the lesion. BrdU and PCNA immunopositive nuclei were rarely observed within non-injured tissue sections, where cell proliferation was limited and generally restricted to the SVZ and pial covering, with occasional labelled nuclei distributed elsewhere in the brain parenchyma (data not shown). By 7 DPI, both BrdU and PCNA labelling was distributed in a broad zone surrounding the injury site and extending into the corpus callosum (Figure 5.2D and E) and SVZ (Figure 5.2F and G), co-localising with nestin.

To determine the phenotype of proliferating cells, tissue sections were double immunolabelled for either BrdU, in animals administered with BrdU at 1 DPI, or nestin, in addition to markers for astrocytes (GFAP), microglia/macrophages (ferritin) and neurons (NF-M, α-internexin, calretinin and parvalbumin) (Figure 5.3). Neuronal markers were selected to represent pyramidal neuron (NF-M and α-internexin) and cortical interneuron (calretinin and parvalbumin) populations. Examination of injured tissue at 7 DPI showed that both BrdU and nestin immunolabelling co-localised with expression of GFAP (Figure 5.3A and B). Additionally, a number of ferritin positive

microglia/macrophages were double labelled with BrdU (Figure 5.3C) indicating they were mitotically active following injury. Contrary to the widespread colocalisation between nestin and GFAP, nestin immunoreactivity was not localised within ferritin-immunopositive activated microglia/macrophages (Figure 5.3D). Furthermore, BrdU and nestin immunoreactivity was absent from all neuronal populations examined within peri lesion tissue, including those immunopositive for NF-M (Figure 5.3E and F), alpha-internexin (Figure 5.3G and H), calretinin (Figure 5.3I and J) and parvalbumin (Figure 5.3K and L).

5.3.3 Pyramidal neurons respond to injury with axonal sprouting by 7 DPI

Cortical sections, cut in a parallel/horizontal plane to the pial surface through the injury site at 7 DPI, were immunolabelled with a range of neuronal markers to examine the neuronal response to injury. The antibody cocktail to phosphorylated neurofilaments, SMI312 (Figure 5.4A and B), and the intermediate neurofilament marker, α-internexin (Figure 5.4C and D) demonstrated that, although no neurogenesis had occurred, neuronal sprouting of resident neuronal populations characterised the response to injury at 7 DPI (Dickson et al., 2005). SMI312 and α-internexin has been previously reported to be expressed in pyramidal neurons, albeit not completely exclusively (Masliah et al., 1993; Dickson et al., 2005). Immunoreactivity for both SMI312 and α-internexin had increased around the injury site (Figure 5.4A and C respectively), and high power confocal images revealed fine sprout like structures within the injury lesion (Figure 5.4B and D, arrows, respectively). However, immunolabelling with calretinin, a calcium binding protein that labels a subset of interneurons exclusively, was confined to the intact tissue, with a discreet injury border (Figure 5.4F), these calretinin immunopositive neurons did not exhibit a sprouting response (Figure 5.4F).

5.3.4 Calretinin neurons show dendritic remodelling at 14 DPI

At 14 DPI, calretinin labelling in the injured rat brains (Figure 5.5A), and the injured thy1 GFP mouse brains (Figure 5.5B), confirmed that a tissue core had formed around the injury site (Figure 5.5A and B, dotted lines). Immunolabelling for calretinin demonstrated a distinguishable neuronal difference between injured and control sham-

injured brains. At 14 DPI the horizontally projecting dendrites of calretinin labelled neurons (Figure 5.5A, arrows) differed from their sham-injured controls (Figure 5.5C). The dendrites of calretinin labelled cells, at 14 DPI, were radially orientated around the lesion site. Expression of GFP in a subset of pyramidal cells at 14 DPI (Figure 5.5B), in comparison to contralateral control sham-injured sections (Figure 5.5D), demonstrated no differences in dendrite alignment between the injured and control sham-injured sections, albeit there was an increase in non-specific immunoreactivity around the injury site.

5.3.5 Calretinin neurons had a significant change in dendritic orientation around the injury site at 14 DPI

To quantitate injury-induced changes in the dendritic arbor orientation of calretinin labelled interneurons, horizontally projecting dendrites were visualised and their orientation relative to the injury site and control sham-injury site determined. All calretinin immunolabelled cells within the four 20x fields of view around the injury were included in analysis for both injured and control sham-injured sections. All dendrites intersecting a circumferential point 80μm from the centre of the neural cell body were analysed. Neurites at this distance corresponded predominately to secondary dendrites that were morphologically elaborate with multiple branch points. Figure 5.6A and B, illustrates a representation of horizontal dendrites of neurons most characteristic for the populations at 14 DPI and control sham injuries, respectively.

To investigate dendritic remodelling in this subpopulation of neurons, the orientation of the individual dendrites for each neuron was determined (see methods). The distribution of the mean angle of all dendrites for each neuron, from control sham-injured and injured animals, relative to the distance the neuron was from the injury site, is represented in Figures 5.6C and D, respectively. This data demonstrates the distinct shift in dendrite angle relative to injury in neurons from injured brains (Figure 5.6D), with a trend for this shift to increase at increasing distances from the injury site, however linear regression demonstrated that this trend was not significant ($p=0.05$). One-way ANOVA of the mean dendrite angle for each injured cell demonstrated a significant ($p<0.001$) decrease in mean dendrite angle at 14 DPI ($n=51$) in comparison

to control sham-injured (n=82) (Figure 5.6E). Furthermore, analysis utilising biaxial θ parameters of circular statistics further identified a difference in plot distributions. Mean dendrite angle for neurons from control animals was 45.509° with a circular standard deviation of 10.542° and for neurons from injured animals 19.065° with a circular standard deviation of 17.065°.

To confirm that the alteration in dendrite orientation was not a result of either dendrite damage or growth, the mean dendrite length and mean dendrite number was quantified. One-way ANOVA demonstrated no significant ($p>0.05$) difference in the mean number of dendrites between the injured and control sham-injured brains, (mean=2.97, SD=+/-1.90 for control sham-injured and mean=2.87, SD=+/-2.16 for 14 DPI). Mean neurite length for all primary, secondary and tertiary dendrites and other dendrites (with more than three branch points) for each interneuron around the injury site in comparison to control, was also analysed. The number of neurites that branched at more than three points was minimal (data not shown). There was no significant ($p>0.05$) difference in mean dendrite lengths for primary, secondary and tertiary dendrites, analysed with a one-way ANOVA (Figure 5.6F).

To determine the directionality of the decrease in mean dendrite angle, the polarity of each neuron investigated was determined. This was analysed by dividing the quantitation zone of each cell into four quadrants (Figure 5.7A). For each dendrite, the quadrant in which the dendrite intersects at 80μm was recorded. The quadrants were divided into proximal, lateral 1 and 2 and distal, relative to the injury site. Two-way ANOVA revealed a significant ($p<0.05$) increase in the mean number of dendrites in the distal quadrant (Figure 5.7B) at 14 DPI in comparison to the control sham-injured. Additionally there was a significant ($p<0.05$) decrease in the mean number of dendrites per neuron in the lateral 1 quadrant (Figure 5.7 C).

5.3.6 The dendrites of pyramidal neurons expressing GFP did not undergo remodelling around the injury site at 14 DPI

To investigate whether the remodelling response was specific to the calretinin positive

interneurons examined or was a stereotypical neuronal response, a subset of pyramidal neurons was also investigated in a transgenic mouse line with GFP expression restricted to this cell type. All GFP positive cells within four 20x fields of view around the injury site were included in analysis for both injured and control sham-injured sections. Again, dendrites intersecting a circumferential point 80μm from the centre of the cell bodies were analysed. Figure 5.8 A and B, illustrates a representation of horizontal dendrites of neurons most characteristic for the populations at 14 DPI and control sham-injuries, respectively. Measurements of mean dendrite angle, for pyramidal neurons in relation to the distance of the cell from the injury site, demonstrated no significant ($p>0.05$) shift in the injured neurons at 14 DPI ($n=86$) in comparison to control sham-injured ($n=46$) (Figure 5.8C,D,E). Additionally there was no significant ($p>0.05$) difference in mean dendrite lengths for primary, secondary and tertiary dendrites, analysed with a one-way ANOVA (Figure 5.8F).

5.4 DISCUSSION

The experimental model used in this Chapter produced a discrete lesion, causing structural damage to the neocortical laminae, but not surrounding or underlying structures (King et al., 1997; 2001). This form of injury generates a stereotypical sequence of changes that ultimately culminate in wound healing, including phases of acute haemorrhage, necrosis and oedema, followed by glial activation, scar formation and wound contraction (Maxwell et al., 1990; King et al., 2001). The present investigations highlight distinct mechanisms of injury-induced neuronal plasticity, which are specific to discrete subpopulations of cortical neurons and progenitor cells. Although there was no evidence of neurogenesis within the damaged cortex, resident pyramidal and inter-neurons undergo contrasting alterations in response to structural injury; pyramidal neurons respond by elaborating fine axonal sprouts into the injury site, whereas interneurons undergo dendritic remodelling, whereby the dendritic arbor becomes reoriented away from the injury site. These responses occurred concurrent with a stereotypical gliogenic response.

Several studies have demonstrated evidence of neuronal proliferation in response to experimental brain injury (for examples see Kernie et al., 2001; Rice et al., 2003), providing support for neurogenesis as a mechanism of brain repair following traumatic brain injury. However, this and other investigations (Holmin et al., 1997; Kuroda et al., 2002; Salman et al., 2004) have indicated that neurogenesis does not lead to nerve cell replacement in the neocortex following an acute focal lesion. Specifically, we observed nestin-labelled cells within the SVZ and corpus callosum by 7 DPI, with nestin immunoreactivity extending up to the injury site. The nestin immunoreactive profiles present within both the SVZ and corpus callosum co-localised with the markers PCNA and BrdU, respectively, indicating they were mitotically active. Double labelling studies indicated that nestin positive cells did not express neuronal marker proteins, however nestin was found to co-localise with the astrocytic marker, GFAP, indicative of a gliogenic, but not neurogenic, response. These results were consistent with the *in vitro* findings in Chapter 4, which additionally did not observe neurogenesis in response to

acute injury. Whether this upregulation of nestin is a result of new cell division or the re-expression of nestin within existing glia (Kronenberg et al., 2005) is unclear.

The lack of post-injury neurogenesis in the neocortex, in the present investigation, may be due to the location and severity of the injury. Many models of cortical lesion generate widespread neural damage (Posmantur et al., 1996), which often directly affects regions associated with high plasticity, such as the SVZ or hippocampus, or at least damages the cortex more extensively. The model utilised in the current investigation, however, specifically targeted the upper layers of the cortical grey matter and thus may not evoke the same signalling mechanisms initiated in more disruptive forms of trauma. The absence of constitutive cortical neurogenesis has been postulated to result from a lack of appropriate micro-environmental cues, rather than a limit of the endogenous precursors themselves (Emsely et al., 2005), and this deficiency may also account for the absence of neurogenesis in the cortex following injury in the present study.

Focal injury to the neocortex evoked a characteristic neurite sprouting response adjacent to the lesion. This sprouting response was likely to be axonal in origin and confined to pyramidal neurons, and was not demonstrated to occur from a subpopulation of interneurons immunopositive for calretinin. This induction of axonal growth after injury may be maladaptive, potentially exacerbating a clinically poor outcome. Both *in vitro* studies) including the findings in Chapters 1 and 2 (McKinney et al., 1997, Chuckowree and Vickers, 2003), and *in vivo* (Salin et al., 1995) experimental models indicate that post-injury sprouting may not always be functionally appropriate, with potential aberrant axonal connectivity possibly contributing to the development of epilepsy following brain lesions such as stroke (Carmichael, 2003). Furthermore, studies have indicated that regenerative axonal sprouts differ from their developmental counterparts, being tipped by bulbous end structures *in vivo* (King et al., 2001; Dickson et al., 2005), and lacking the ability to path find *in vitro* evidenced by an altered growth cone behaviour in mature regenerating growth cones in comparison to developing growth cones in Chapters 1 and 2. Recent research indicates that the anatomical and functional plasticity of surrounding intact cortex, rather than frank regeneration of damaged

neurons, may play a more fundamental role following injury. Hence, understanding the mechanisms underpinning the lack of injury-induced axonal sprouting in interneuron subpopulations may reveal fundamental differences in the intrinsic plastic capabilities of different cortical neuron populations and what implications this may have for brain healing.

Although post-injury axonal sprouting was shown to be specific to pyramidal neurons, interneurons were not without injury-induced remodelling capabilities. This chapter demonstrated a particularly novel property of a subpopulation of interneurons, namely those that were calretinin immunoreactive, to remodel dendritic arbors in response to acute brain lesion. Cortical interneurons within the barrel cortex have previously been shown to have a capacity for morphological plasticity (Lee et al., 2006), however, this was under physiologically normal conditions. Furthermore, previous investigations have suggested that the potential sites for structural plasticity are the horizontal connections within the superficial layers of the cortex (Darian-Smith and Gilbert, 1994; Kaas et al., 1990; Lee et al., 2008). Following acute brain injury in the current study, the dendritic arbor of calretinin positive interneurons was orientated away from the injury site, relative to sham-injured controls. Although it was postulated that this change in dendritic orientation was due to new dendrite growth in response to injury, further analyses of both mean dendrite number and mean neurite length revealed that neither parameter was altered as a result of the injury. Hence the alteration in dendrite alignment was most likely a result of remodelling of existing arbors, such that those close to the injury retracted, while those distal to the injury grew. These findings are consistent with studies demonstrating that GABA positive interneurons exhibit dynamic arbor re-arrangements while pyramidal populations remain stable, under physiologically normal conditions (Frahm et al., 2004).

A subset of pyramidal neurons, specifically those expressing GFP in the transgenic mouse, was also investigated to determine if this remodelling was cell-type specific. While the injury site in these mice had formed a comparable tissue core by 14 DPI, which indicates an active brain response, statistical analysis revealed no significant remodelling in the dendrites of these pyramidal neurons. Thus the dendritic

remodelling observed at around the injury site, in our model of brain injury, may be confined to interneurons, specifically the calretinin positive population, demonstrating a cell-type specific response to injury. These findings are in coherence with studies demonstrating that the GABA positive interneurons exhibit dynamic arbor re-arrangements while pyramidal populations

This Chapter supports the proposal that the adult brain has a frequently unappreciated capacity for cytoarchitectural remodeling and repair following injury. In particular, this Chapter demonstrated a novel cell-type specific response of neuronal populations to acute injury; a subpopulation of pyramidal neurons elaborated axonal sprouts into the injury site whereas a subpopulation of interneurons (calretinin neurons) underwent a reorganization of their dendrites for a distal bias away for the injury site. These distinct responses, within different cortical neuron populations, provide novel insight into the potential of the mature brain for structural plasticity following injury.

6 COMPLETE LOSS OF THE NEUROFILAMENT LIGHT CHAIN PROTEIN INTRINSICALLY REDUCUES THE REGENERATIVE PLASTICTY OF MATURE CNS NEURONS

6.1 INTRODUCTION

The axonal cytoskeleton plays a vital role in axon outgrowth and pathfinding in both development and regeneration (Gordon and Weeks, 2004; Graham et al., 2000). A key component of the cytoskeleton is the neurofilament triplet proteins, which enable elongation of the axons (Walker et al., 2001) and provide mechanical strength to axons by counteracting compressive forces of the local environment (Hoffman et al, 1984; Cleveland et al., 1991; Lee and Cleavland, 1996; reviewed in Lariviere and Julien, 2004). The neurofilament triplet component is predominately comprised of three main neurofilament proteins, NF-L, NF-M and NF-H. The expression of the neurofilaments during neuronal development follows a highly co-ordinated sequence of expression, which is directly related to the maturity of the neuron. During development, NF-L is the first neurofilament triplet protein to be expressed, which is then sequentially followed by NF-M and then NF-H as the neuron matures (Benson et al., 1996). However, the early expression of NF-L can overlap with the other neurofilament proteins, which are expressed early in development, α-internexin and peripherin (Willard and Simon, 1983; Carden et al., 1987; Escurat et al., 1990).

Classically, NF-L was believed to play a pivotal role in the formation of the triplet protein, and importantly, without NF-L, the triplet protein would not form (Carter et al., 1997). Furthermore, lack of NF-L or NF-L dysfunction has been linked to the aetiology of amyotrophic lateral sclerosis and Charcot-Marie-Tooth disease, highlighting the importance of this cytoskeletal component (Beaulieu et al., 2000; Mersiyanova et al., 2000; reviewed in Lin and Schlaepfer, 2006). NF-L forms a core of protein which NF-M and NF-H bind to, forming the triplet protein (Nixon 1993). NF-L enables the formation of this triplet through binding to the protein NDel1 (Nyugen et al., 2004). NDel1 is the mammalian homologue of the *Aspergillus nidulans* NudE, which was initially shown to be involved in microtubule migration. NDel1 binds directly to NF-L,

and indirectly to NF-H and this binding facilitates the triplet formation (Nyugen et al, 2004).

Neurofilaments have traditionally been thought to be obligate heterodimers with a subunit stoichiometry of 4:2:1 (NF-L:NF-M:NF-H) (Scott et al., 1985). However, recent studies have challenged this traditional stoichiometry, specifically, α-internexin has now been shown to closely associate to the neurofilament triplet protein *in vivo* (Yuan et al., 2006). Indeed, the purification of neurofilament quadruplets from optic nerve and spinal cord suggests a new stoichiometry of 4:2:2:1 (NF-L: α-internexin: NF-M: NF-H) (Yuan et al., 2006). The current Chapter endeavoured to investigate the effect a total knock out of NF-L would have on the formation of the triplet protein. Furthermore, while the effect such a loss of NF-L has on regeneration has been investigated within the peripheral nervous system (McLean et al., 2005; Toth et al., 2008), few studies have looked at the CNS. To investigate the role of the neurofilament triplet protein in regeneration a transgenic model of mice with a targeted disruption of the NF-L gene was utilised (Zhu et al., 1997; Zhang et al., 2002). This Chapter investigated the cytoskeletal alterations, specifically the reactive and regenerative changes that characterise the axonal response to physical injury in neurons lacking NF-L. An *in vitro* model of axonal transection in mouse cortical neurons was established, and used in conjunction with the focal neocortical injury model described in Chapter 5. The neuronal characteristics and response of regenerating axons was analysed using multi-labelling immunohistochemistry, immunocytochemistry and molecular techniques.

6.2 MATERIALS AND METHODS

6.2.1 Neuronal culture and *in vitro* model of axonal transection

Primary dissociated cortical cultures were prepared as previously described (Chapter 2.1), with alterations in the protocol. A transgenic homozygous mouse colony with a complete knock out of NF-L on a black C57 background was utilised for these studies (kind donation from Dr Mala Rao, Dr Stephen Ginsberg and Professor Jean-Pierre Julien) compared to wild type mice derived from a black C57 mouse colony. Cortical neurons were derived from E14 NF-L-/- (Zhu et al., 1997) and C57/bl6 (wildtype) mouse embryos, cultured separately. Axonal transection was carried out on relatively mature cultures (15 DIV), when a reduction of developmental proteins coincided with the expression of proteins correlating to maturity of the neuron and its processes (Figure 6.1). Four injuries were made per coverslip, ensuring that complete axonal transection had occurred as described in Chapter 2.2, leaving a cell free lesion (approx 50 to 150μm wide). Cultures were fixed and examined at 24 hrs PI.

6.2.2 Immunocytochemistry and Immunohistochemistry

Cultures (3, 7 and 15 DIV and 24 hrs post-injury in 21 DIV cultures) were fixed in 4% PFA/0.01M PBS for 30 mins at room temperature on an orbital shaker and processed for immunocytochemistry as described in Chapter 2.4 (for antibody details see Table 2.1). For immunohistochemistry NF-L-/- mice animals were terminally anaesthetised (pentabarbitone sodium, 140mg/kg) and transcardially perfused with 4% PFA/0.01M PBS, following a 0.01M PBS clearing procedure. Non-injured, age-matched wildtype controls corresponding to the 1, 7 and 14 DPI time-points (n=5 animals per time-point) were also perfused. Brain sections (50μm vibratome) were collected coronally and also horizontal to the pial surface, through the injury site, from control and experimental animals and were immunohistochemically labelled in single as well as double-labelling combinations, with a range of cell specific antibodies (Table 2.1), as described in Chapter 2.4. Alexa Fluor® 594 and 488 (1:1000, Invitrogen) secondary antibodies (diluted in 0.01M PBS) were applied for 90 min, at room temperature. Immunolabelling was visualised using a Leica (Germany) DMLB2 upright fluorescent microscope.

6.2.3 Immunoblotting and Coimmunoprecipitation

Coimmunoprecipitation and immunoblotting was performed on relative mature neurons (15 DIV). Neurons were grown at a density of $4x10^8$ in 25 mm flasks (Iwaki, Japan) and harvested at 4°C in mammalian tissue lysis buffer (Sigma, USA), supplemented with a cocktail of protease inhibitors and phosphatase inhibitors. Coimmunoprecipitation was performed using the Pierce (USA) coimmunoprecipitation kit, as per manufacturers instructions. Antibody concentrations and elution time were optimised. For immunoblotting, initially, proteins were separated by SDS-PAGE using a Mini-PROTEAN® II (Bio-Rad, USA) system. 8.5% 0.375M Tris-HCl separating gels were used. The separating gel was overlayed with a 4% 0.125M Tris-HCl, pH 6.8 stacking gel. Samples were prepared by dilution in 2x Laemelli sample buffer (1:2), which were then heat treated at 90°C for five minutes and incubated on ice for five minutes. A broad range, pre-stained rainbow molecular weight marker (Bio-Rad, USA) was included in each separation to facilitate protein molecular weight determination. The gel was run at 100V for the duration of the separation.

After separation, gels were stained for total protein with Coomassie stain, or used for immunoblotting, utilising a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, USA). Prior to transfer, gels were equilibrated in transfer buffer for 15 minutes at room temperature, on an orbital shaker. The gel 'sandwich', containing a nitrocellulose membrane (Bio-Rad,USA) and the gel, was inserted into the transfer cell. Protein transfer was performed overnight at 4°C, 30V. The nitrocellulose membrane was washed three times with tris buffered saline with tween (TTBS) for five minutes, before blocking with TTBS / 5% skim milk powder overnight. Blocking solution was removed by washing with TTBS. Primary antibodies were diluted in TTBS and incubated with the membrane overnight at 4°C on an orbital shaker. Membranes were washed three times with TTBS for ten minutes, then incubated with species appropriate horseradish peroxidase conjugated secondary antibodies (1:1000 to 1:2000, DAKO, Denmark), diluted in TTBS, for 1.5 hours at room temperature, on a shaker. After this, membranes were washed three times with Tris buffered saline (TBS), for ten minutes.

Protein bands were labelled using an ECL™ chemiluminescence system (Sigma, USA), according to the manufacturers instructions. Bands were visualised on the ChemCapture 5000 and ChemCapture program.

6.3 RESULTS

6.3.1 Mouse cortical neurons develop *in vitro* and become relatively mature by 15 DIV.

The experiments included in this chapter utilised cultured cortical neurons derived from embryonic mice instead of rats and thus it was first necessary to determine if cultured mouse neurons follow a similar time-course of maturity to that established for cultured rat neurons. Cortical neurons were derived from E14 mouse pups, this earlier time-point in comparison to rats yielding a greater proportion of viable neurons. Cortical neurons were plated as a monolayer on a poly-L-lysine substrate and the expression of maturity related markers investigated (Figure 6.1). Immunocytochemistry indicated that mouse cortical neurons became relatively mature by 15 DIV. By 7 DIV, neurons were clearly polarised with MAP-2 positive dendrites and a Tau positive axon (Figure 6.1A). However, an increase in expression of MAP-2 was clearly evident by 15 DIV (Figure 6.1B). GAP-43 was enriched in cell bodies, axons, dendrites and growth cones (inset) at 7 DIV (Figure 6.1C). By 15 DIV the immunoreactivity of GAP43 was decreased, with minimal immunoreactivity in the axons and dendrites (Figure 6.1D). Colocalisation of βIII tubulin and the synaptic vesicle marker synaptophysin at 7 DIV indicated that synaptophysin was diffusely localised in the neurites. By 15 DIV synaptophysin was localised alongside the dendrite shafts (inset), demonstrating that synaptophysin had moved out of the dendrite shafts into the synapses, indicating mature synapses were present (King et al., 2006).

6.3.2 NF-L-/- cortical neurons develop polarised neurites *in vitro* by 3 DIV

Light microscopy and immunocytochemistry was performed on cortical neurons derived from wildtype control and NF-L-/-, to determine whether NF-L-/- cortical neurons undergo characteristic neuronal development *in vitro*. NF-L-/- cortical cultures adhered to

the substrate and had extended neurites by 3 DIV (Figure 6.2B). These neurites were tipped with characteristic growth cones (Figure 6.2A and B inset). Immunolabelling for the microtubule associated proteins tau and MAP-2 at 3 DIV, demonstrated that both control and NF-L-/- neurons had developed polarised neurites, with clearly defined dendrites (red) and one longer axon (green) (Figure 6.2C and D respectively). By 15 DIV (Figure 6.3), wildtype and NF-L-/- neurons demonstrated a comparable increase in the expression of MAP-2, when colocalised with Tau (Figure 6.3A and B respectively) when compared to 3 DIV (Figure 6.2C and D). Labelling with an antibody directed NF-L confirmed that wildtype neurons expressed NF-L at 15 DIV (Figure 6.3C) while the neuronal cultures derived from NF-L-/- mutants had no immunoreactivity for this protein (Figure 6.3D).

6.3.3 The expression profile of neuronal intermediate filaments in developing and relatively mature NF-L-/- cortical neurons differs from wildtype cortical neurons both *in vitro* and *in vivo*

To investigate the expression of neuronal intermediate filaments in the NF-L-/- cortical neurons in comparison to wildtype cortical neurons, immunocytochemistry was performed on cultured neurons at 7 DIV and 15 DIV NF-L-/-. At 7 DIV NF-M and α-internexin were present throughout axons in both wildtype and NF-L-/- cortical cultures (Figure 6.4A-D). NF-M immunoreactivity was decreased within NF-L-/- neurons (Figure 6.4B) in comparison to wildtype control (Figure 6.4A). However, there was an increased immunoreactivity for α-internexin in NF-L-/- neurons at 7 DIV, in comparison to wildtype control (Figure 6.4A and B). Dual labelling immunocytochemistry utilising NF-M and α-internexin antibodies at 7 DIV demonstrated that both wildtype (Figure 6.4E) and NF-L-/- (Figure 6.4F) neurons extended axons that expressed NF-M and α-internexin independently. Additionally, at 7 DIV, there were axons present that expressed both proteins (Figure 6.4E and F, arrows). Immunolabelling of NF-M and α-internexin at 15 DIV demonstrated that wildtype axons were predominately immunoreactive for either NF-M or α-internexin exclusively (Figure 6.5A, arrows). NF-L-/- axons were immunopositive for both NF-M and α-internexin (Figure 6.5B, arrowheads). The decrease in NF-M expression and increase in α-internexin expression in cultures derived

from NF-L-/- mice relative to wildtype mice was confirmed through a western immunoblot, from cells harvested at 15 DIV (Figure 6.5C).

To confirm the difference in expression of neuronal intermediate filament proteins *in vivo*, dual labelling immunohistochemistry utilising NF-M and α-internexin antibodies was performed in neocortical neurons from coronal sections of wildtype and NF-L-/- mice. In wildtype mice, axons of neocortical neurons similarly demonstrated immunoreactivity for either α-internexin or NF-M exclusively (Figure 6.5D, arrows). In the NF-L-/- axons however, were primarily immunopositive for both NF-M and α-internexin (Figure 6.5E, arrowheads) confirming the widespread colocalisation of NF-M and α-internexin observed *in vitro* in cultures derived from these animals (Figure 6.5A and B).

The immunoreactivity profile of a range of antibodies was investigated in the neocortex of wildtype and NF-L-/- coronal sections. MAP-2 labelling of the neocortex of wildtype and NF-L-/- sections demonstrated no detectable differences between the conditions (Figure 6.6A and B). Nuclear yellow staining (wildtype and NF-L-/-, Figure 6.6 B and C respectively) indicated that there was a similar distribution of nuclei throughout the neocortex. Immunohistochemistry for the interneuronal marker parvalbumin (wildtype and NF-L-/-, Figure 6.6E and F respectively) also did not reveal any distinguishable differences in the immunoreactivity pattern within the neocortex. Parvalbumin immunoreactivity was widespread throughout the neocortex, with a high density of parvalbumin immunoreactive neurons in layer V (Figure 6.6E and F). Immunohistochemical labelling for the neurofilament markers SMI312, NF-M and α-internexin in wildtype sections was predominately within the pyramidal cortical layers, specifically layer III and V (Figure 6.7A,C and E respectively). SMI312 immunoreactivity was decreased within the NF-L-/- neocortex and the distinct immunoreactive layer patterning demonstrated in the wildtype neocortex was not present (Figure 6.7B). NF-M immunoreactivity was also decreased within the NF-L-/- cortex (Figure 6.7D) in comparison to wildtype control (Figure 6.7C), with no pattern of immunoreactivity corresponding to cortical layers. Cortical layers within NF-L-/- neocortex (Figure 6.7F) were also not distinguishable with α-internexin immunoreactivity

in comparison to wildtype control (Figure 6.7E), despite an increase in immunoreactivity within the NF-L-/- neocortex (Figure 6.7E and F).

6.3.4 The neurofilament associated protein NDel1 is present and associates with α-internexin in the absence of NF-L

The deletion of NF-L-/- utilised by these studies resulted in an increase in α-internexin within the neurons. Thus it was hypothesised that α-internexin may be compensating for the NF-L loss and allowing the formation of the neurofilament triplet protein. Furthermore, it was hypothesised that NDel1, in the absence of NF-L, may bind to α-internexin to facilitate the formation of the triplet protein. The expression of NDel1 in NF-L-/- and wildtype mice and it's interaction with α-internexin was investigated using immunohistochemical techniques *in vivo* and *in vitro* and coimmunoprecipitation of protein derived from cultured neurons (Figure 6.8E). *In vitro,* NDel1 immunoreactivity was present at 15 DIV in both wildtype and NF-L-/- neurons (Figure 6.8A and B). A similar immunoreactivity profile between wildtype and NF-L-/- neurons was also observed *in vivo* with NDel1 immunoreactivity being confined to axonal shafts (Figure 6.8C and D. Alpha-internexin was immunoprecipitated from 15 DIV wildtype and NF-L-/- neurons. NDel1 was immunoprecipitated with α-internexin in the NF-L-/- neurons, but did not co-precipitate with α-internexin in the wildtype neurons (Figure 6.8D).

6.3.5 NF-L-/- mice respond actively in response to a focal neocortical injury, which culminates in axonal sprouting and the formation of the glial scar.

The response of the NF-L-/- to acute focal neocortical injury was investigated utilising the model previously described in Chapter 5. To investigate the glial response to acute injury in the mouse neocortex sections were immunolabelled with GFAP. There was minimal GFAP immunolabelling in the uninjured neocortex of wildtype mice (Figure 6.9A). NF-L-/- neocortex in contrast, had a population of resident astrocytes that expressed GFAP under physiologically normal conditions (Figure 6.9B, arrows). This increase in GFAP had been previously described within the spinal cord of these mice (McLean et al., 2005). Immunolabelling for GFAP at 4 DPI in both NF-L-/- and wildtype

mice demonstrated that the injury and peri-lesion site had now become filled with reactive astrocytes (Figure 6.9C and D). By 7 DPI (Figure 6.9E and F) GFAP labelling indicated that an astrocyte filled glial scar had formed at the injury site. By 14 DPI (Figure 6.9G and H) a significant proportion of the tract had closed together leaving a central dense GFAP labelled core. There were no differences between the injury response of the wildtype and NF-L-/- neocortex, when visualised with GFAP immunoreactivity. To determine the sprouting response in both conditions, sections were immunolabelled with the phosphorylated neurofilament marker SMI312. At 4 DPI, SMI312 labelling demonstrated that NF-L-/- neurons responded actively to the focal injury, with fine sprouts present within the injury site similar to those present in wildtype mice (Figure 6.10A and B, arrows).

6.3.6 There was a significant decrease in the number of NF-L-/- sprouts within the injury site *in vitro*

To further investigate differences in the wildtype and NF-L-/- regenerative response, the neuronal response to injury was investigated in relatively mature neurons *in vitro.* The neuronal network of dendrites and axons in cultures at 15 DIV were completely transected, leaving a clear injury site (Chuckowree and Vickers 2003; Haas et al., 2004). Injured neurons were fixed at 24 hrs PI and the axonal regenerative response investigated using tau immunocytochemistry (Figure 6.11). Fine sprouts were transversing the injury at 24 hrs for both wildtype and NF-L-/- neurons; these fine sprouts were tipped with characteristic growth cones (Figure 6.11A and B respectively, arrows). There was no significant difference in mean sprout length for wildtype (68.76+/-3.44μm) and NF-L-/- (56.19+/-15.47μm) regenerative sprouts at 24 hrs PI (Figure 6.11C). However, there was a significant ($p<0.05$) difference between mean numbers of sprouts per injury (Figure 6.11D), was significantly less NF-L-/- (4.79+/-0.58) sprouts within the injury site in comparison to wildtype control (10.47+/-1.11).

6.4 DISCUSSION

Primary neuronal cultures have traditionally been derived from rat embryos; therefore these protocols are well established in the literature (Dotti *et al*., 1988; Goslin *et al*., 1990; Bradke and Dotti, 1999; Haas *et al*., 2004; King *et al*., 2006). Increasingly researchers are using transgenic mice to model aspects of certain diseases and to understand the biological function of specific proteins, thus more recent studies report using cultured neurons derived from these transgenic mice (Lee and Zheng, 2008; Ashe and Zahs 2010; Luebke et al., 2010) These protocols are, however, less well established and require optimisation depending on the background of the strain. Cortical cultures derived from wildtype (black C57) neurons utilised in this Chapter became relatively mature by 15 DIV. While the cause for this decrease in days *in vitro* required by mouse neurons to mature in comparison to neuronal cultures derived from rats has not been directly determined, it is most likely reflective of variations in the gestation time between species.

Development of NF-L-/- neurons in culture followed a similar time-course to the wildtype neurons, with appropriately polarised neurites extended by 3 DIV, indicating that NF-L is not required for neurite initiation. This confirms the hypothesis that initial neurite growth and growth cone formation is governed by the microtubules and microfilaments (Dent and Kalil 2003; Gordon and Weeks 2004). However, neurofilaments are required for appropriate neuronal polarisation (Lee and Cleveland, 1996). This thesis demonstrates that appropriate polarisation is not reliant upon NF-L, indicating a possible functional redundancy between the neuronal intermediate filaments in the initiation of polarisation. Both during development and in relatively mature neurons, there were distinct differences in immunoreactivity for the neurofilaments markers NF-M and α-internexin.

Previous studies have indicated that α-internexin may, under certain circumstances, form part of the neurofilament triplet protein. Alpha internexin has been shown to be associated closely to NF-M in the optic nerve (Yuan et al., 2006), and in the absence of NF-L and NF-H, is required for NF-M transport (Yuan et al., 2003). Furthermore, both

NF-M and α-internexin immunoreactivity is in a linear arrangement continuously down the axon, indicative of neurofilament triplet formation (Nguyen et al., 2004). In the current study NF-M immunoreactivity was decreased in NF-L-/- mice with a relative increase in α-internexin both during development and after reaching maturity, confirmed with immunoblotting (Zhu et al., 1997; Zhang et al., 2002). Importantly, NF-L-/- neurons both *in vitro* and *in vivo* demonstrated a complete colocalisation for NF-M and α-internexin, indicating that NF-M and α-internexin associated together within the NF-L-/- neurons. Under normal conditions NF-L expression outnumbers α-internexin 2:1 (Yuan et al, 2006). Furthermore, it has been suggested that there may be a functional redundancy within the neurofilament triplet proteins, with deletion of one protein compensated for by another neurofilament (Rao et al., 2003; Yuan et al., 2003; Yuan et al., 2006). The relative increase in α-internexin immunoreactivity and expression, coupled with the exclusive colocalisation with NF-M, indicates that α-internexin in fact may be compensating for the loss of NF-L, providing further evidence that α-internexin is potentially able to form the triplet protein.

Additional evidence for the role of α-internexin in the assembly of the triplet protein was demonstrated through coimmunoprecipitation of NDel1 with α-internexin. NDel1 has been shown to facilitate the formation of the triplet protein, through direct binding to NF-L, and indirect binding to NF-H (Nyguen et al; 2004). In the current investigation, only in the absence of NF-L, did NDel1 bind to α-internexin. This activity titration of NDel1 has previously been observed in the peripheral nervous system (Toth et al., 2008). In dorsal root ganglia of NF-L-/- mice, NDel1 was shown to bind to vimentin, another intermediate filament family member (Toth et al., 2008). The interaction of α-internexin and NDel1, in conjunction with the increase in expression of α-internexin, observed in the current study suggests that there may be a functional redundancy of α-internexin and NF-L, within the triplet protein. However, the specific binding affinity for α-internexin with NDel1remains to be fully elucidated, and would provide significant insight into the role of α-internexin in the triplet protein.

Behavioural analysis of NF-L null mice has shown that by 6 months NF-L-/- mice have mild sensorimotor dysfunction and spatial deficits (Dubios et al., 2005). The current

study observed discreet differences within the organisation of neocortical immunoreactivity of NF-L-/- mice in comparison to wildtype control. The neocortex is a highly specialised structure, which is organised into 6 layers, or laminae (Silberberg et al., 2005). In the wildtype control, labelling for the neurofilament markers was intensified within the pyramidal layers of the neocortex. This layer specificity was not present within the NF-L-/- neocortex, which may be due to the alterations of the expression of neurofilament proteins in the different cell types of the cortical laminae. The loss of layer immunoreactivity profiles was restricted to the neurofilament proteins investigated, and was not present within the interneuronal populations. The loss of layer specificity may affect the neural processing within NF-L-/- mutants as interactions between cortical pyramidal cells and interneurons facilitate highly integrated cortical processing (Douglas and Martin 2004).

NF-L-/- null mice underwent a characteristic active response to injury, which culminated in axonal regeneration and the formation of the glial scar. As previously addressed in the current thesis, following injury neurons can attempt to regenerate in two ways. This is either through the frank regeneration of the directly injured neurite, or collateral sprouting of surrounding, uninjured neurites. A classical hallmark of many neurodegenerative diseases is the disruption and accumulation of neurofilaments within the cell body and axons (reviewed in Liem and Messing 2009). Models of TBI have demonstrated that neurofilament loss and/or compaction are a common consequence of injury and neurofilament disruption following injury has severe effects on neuronal structure and function (Posmantur et al., 2000). Importantly, however, neurofilament proteins may play an important role in axonal regeneration as in animal models where robust axonal regeneration occurs, the regenerating axonal sprouts are neurofilament rich (Jacobs et al., 1997). The current Chapter demonstrated that the neurofilaments, specifically NF-L are also involved in axonal regeneration in the CNS. NF-L-/- neurites were able to mount a regenerative response, which culminated in sprout like structures transversing the injury site, both *in vitro* and *in vivo*. There was no significant difference in the mean length of these regenerating neurites, in comparison to control. However, there was a significant reduction in the number of sprouts within the injury. Previous investigations have shown that mice lacking NF-L have a reduced

ability to regenerate in the PNS (Zhu et al., 1997). However, this reduced ability to regenerate may be a delayed response, as by 2 months post-injury there were similar numbers of myelinated axons in the NF-L-/- mice and wildtype controls (Zhu et al., 1997). In the current study, 24 hrs PI represents a relatively prolonged time-point *in vitro*, suggesting that the delayed response to injury which has been demonstrated in the PNS, may not account for the reduction in regeneration within relatively mature CNS neurons. The demonstrated reduction of sprouts within the injury site could either be a result of less injured neurites frankly regenerating, or a decrease in the plasticity of the surrounding uninjured neurites (reviewed in Chuckowree et al., 2004). Utilising both *in vitro* and *in vivo* models, the current Chapter demonstrated that NF-L deletion resulted in a decreased regenerative ability, indicating that regeneration within the mature CNS is intrinsically driven (Li *et al*., 1995; Blackmore and Letourneau 2006).

A novel interaction between α-internexin and the NF-L binding protein, NDel1 was demonstrated within NF-L-/- neurons. This interaction, in conjunction with immunohistochemical evidence, suggests that α-internexin may be forming the functional neurofilament triplet protein in the absence of NF-L. However, while α-internexin may be compensating for NF-L, the compensatory intermediate filament structure present demonstrated an attenuated response to injury both *in vitro* and *in vivo*, indicating that presence NF-L, and most likely the proper formation of the neurofilament triplet protein, is an intrinsic requirement for axonal regeneration and plasticity.

7 DISCUSSION

Traumatic brain injury remains a leading cause of death and disability in persons under the age of 45 years, in developed nations (Adams, 1995; Egeler-Peerdeman, 1993; Povlishock, 2005; Maas et al., 2008). TBI has such an impact primarily due to the limitations on repair of the mature CNS following injury. Current research efforts are focused upon finding a cure or therapeutic intervention that can overcome the brains' limited capacity to regenerate in order to prevent or correct functional deficits. However, strategies for intervention are difficult as the pathobiology of the brain following injury involves a complex sequence of events that remains to be fully elucidated, which is confounded by individual patient differences (reviewed in Lingsman et al., 2010). Understanding the cellular mechanisms underlying the capacity of the damaged brain to attempt repair from injury is, thus, vital for devising effective future therapies to treat brain injury. The inability for the brain to repair has been commonly attributed to the non-permissive nature of the mature CNS to functional neuronal regeneration. However, removal of this environment does not induce spontaneous robust neuronal regeneration (eg Li et al., 1995; Blackmore and Letourneau 2006) indicating that the lack of regeneration in the mature CNS is also an intrinsic limitation. This failure to regenerate is restricted to the mature CNS, with both the immature CNS and mature PNS demonstrating a robust regenerative capacity following injury.

The loss of brain function and the resulting ongoing disability that occurs following TBI is ultimately a result of disconnections in the intricate neuronal network of the brain, this disconnection being either a result of overt neuron loss or neuron damage, the latter particularly affecting the axon. Ultimately, recovery will therefore require the replacement and recovery of the dead and dying cells within the injury, the frank regeneration of injured axons and/or compensatory plasticity of intact pathways to reinstate the interrupted connections. This thesis addressed these three main pathways for adaptation to injury in endeavouring to characterise the response of the mature brain to injury, specifically focussing on cell proliferation that occurs as a response to injury, axonal injury and the regenerative response and the compensatory plasticity that can

occur in the brain following acute injury. This thesis utilised a range of both *in vitro* and *in vivo* methodological approaches, focusing on specific neuronal subpopulations within the neocortex following injury, to provide novel insight into how the mature mammalian brain responds to injury.

Whether brain injury evokes a neurogenic response in the neocortex that contributes to functional recovery remains contentious (Kornack and Rakic, 2001; Gould and Gross 2002; Rakic, 2002; Koketsu et al., 2003). In the current thesis, data obtained from both *in vitro* and *in vivo* experiments clearly show that neurogenesis does not occur in response to a variety of forms of injury. The lack of neurogenesis could be due to the absence of extrinsic signalling pathways (Emsely et al., 2005), for example, due to the lack of these factors in *in vitro* preparations, or the absence of injury extending to germinal regions in the *in vivo* model. Indeed, other studies involving relatively discreet CNS injury also found no evidence of neurogenesis (Holmin et al., 1997; Kuroda et al., 2002; Salman et al., 2004). Hence, the absence of neurogenesis in both *in vitro* and *in vivo* models investigated may be due to the absence of appropriate micro-environmental cues, rather than a limit of the endogenous precursors themselves (Emsely et al., 2005). This indicates that while neurogenesis has the potential to play a vital role when there is extensive damage and cell death, it may not be an ideal target for intervention in forms of TBI that do not induce widespread ipsilateral and contralateral injury. Hence, focal brain injuries may be more dependent upon axonal sprouting and extant neuronal remodelling to achieve some degree of functional improvement.

Axons are highly vulnerable to TBI due to their unique size, morphology and high metabolic activity. The current study investigated the active response of axons to a variety of models of primary injury, both *in vivo* and *in vitro*. Acute neocortical injury *in vivo* and both models of axonal injury *in vitro* clearly demonstrate that the mature neurons can survive a traumatic insult and some neurons possess a capacity to attempt regeneration following injury. These fine neurites correspond to sprouting axons that transverse experimental injury sites, and have been previously documented in both *in vitro* (Chuckowree et al., 2003; Chung et al., 2004) and *in vivo* (King et al., 2000)

models. Studies presented in this thesis further characterised these sprouts to be axonal of origin and no evidence of comparable dendrite spouting was found indicating that sprouting in response to injury was axonal specific.

Axonal injury has been shown to involve a specific sequence of alterations, suggestive of a stereotypical reaction to injury (King et al., 1997; 2000; 2001; Chen et al., 2003a). Studies performed *in vitro* in the current thesis, included in Chapters 3 and 4, demonstrated that this axonal regenerative attempt occurred regardless of the mode of primary injury. Thus the axonal regenerative response can be considered a stereotypical intrinsic response to injury, which is neuron type-specific and not necessarily dependent upon the mode of injury, or severity, supporting previous investigations that have demonstrated that while an increase in injury severity increases the response time of regenerative sprouts, the ultimate outcome was the same (Levent et al., 2007). Additionally, the similarities in the neuronal response after the two different types of primary injury *in vitro*, axonal transection or complete axonal shearing, indicates the reproducibility of the response over a range of different models of traumatic injury. NF-L-/- studies provided further evidence of the intrinsic nature of the regenerating sprouts. Deletion of NF-L resulted in a significant reduction in the sprouting response *in vitro*, indicating that there was a reduction in the intrinsic capacity of axons for structural remodelling related to the absence of a normal neurofilamentous network. This loss of structural plasticity as a result of NF-L deletion following injury has been previously demonstrated following PNS injury (McLean et al., 2005; Toth et al., 2008). Thus, this thesis has demonstrated that this intrinsic reduction in regenerative ability related to the absence of NF-L also pertains to the CNS.

The limited axonal outgrowth of regenerating neurites in comparison to development observed in this thesis may be responsible for the lack of robust CNS axonal extension that has been observed in permissive environments (Li et al., 1995; Blackmore and Letourneau 2006). However, while robust axonal regeneration occurs in the PNS, misdirection of regenerating axons has been investigated in the PNS and may account for the poor functional recovery (eg de Ruiter et al., 2008). This thesis suggests that misdirection of regenerative sprouting may not be limited to the PNS. In this regard,

the regenerative axonal sprouts in both *in vitro* and *in vivo* models of CNS injury were tipped with growth cone-like structures, but these lacked key morphological and dynamic characteristics of developing neurites, which indicates an impeded path finding ability and/or an inability to appropriately branch, both of which would contribute to a poor outcome post-injury. Furthermore, this thesis demonstrated that regenerative sprouts were unresponsive to growth factors, despite the presence of relevant receptors. This also supports the proposal that CNS neuronal regeneration lacks intrinsic capacity to respond to guidance cues in the same fashion as in initial brain development. In this regard, intervening after injury to create a more growth permissive environment would still not likely be advantageous if the regenerating axons are not able to respond to growth factors and other cues for guiding appropriate synaptogenesis. Thus, a potentially more suitable way to induce a robust axonal extension may be to alter the intrinsic growth state of a mature injured axon.

Another way neurons can respond to injury and recover following injury is through the induction of compensatory morphological or structural plasticity in either the injured neurons or surrounding non-injured neurons. Collateral sprouting from uninjured axons has been indicated as a potential adaptive response to injury (Deller and Frotscher, 1997), and may be stimulated due to availability of vacant synaptic territory within the injury site (McKinney et al., 1997). Previous investigations have demonstrated that the outgrowth of dendrites following injury can be correlated to axonal rearrangement and, interestingly, that alterations in dendritic morphology are associated with synaptic modifications (reviewed in Macias, 2008). This thesis demonstrated that not only is the axonal sprouting response cell type specific within the cortex, but also that specific subpopulations of interneurons have a remarkable capacity for structural dendrite remodelling, directed towards the intact cortex. This novel finding may provide further powerful insight into how the structural plasticity of the CNS and how this may contribute to adaptation to brain injury. Manipulation of the plastic potential of these inhibitory neurons around the injury site may provide a novel therapeutic window for rewiring the intact connections to establish new pathways for the functional deficits lost as a result of TBI.

7.1 CONCLUSIONS

The adult brain has a frequently unappreciated capacity for cytoarchitectural remodeling and repair following injury. This thesis provided significant insight into why particular forms of regenerative neuronal changes are limited or aberrant, as well as how the response may be neuronal subtype-specific in the neocortex. In summary, the findings of the thesis indicate that induction of axonal sprouts into the injury site following trauma may not result in a functionally appropriate or desirable outcomes. Instead the compensatory plasticity around the injury site, directed to the uninjured or less damaged cortex, may provide a more functionally appropriate therapeutic target for future directions.

8 REFERENCES

Abrous DN, Koehl M, Le Moal M. 2005. Adult neurogenesis: from precursors to network and physiology. Physiol Rev 85(2):523-69.

Adams PF, Schoenborn CA, Moss AJ, Warren CW, Kann L. 1995. Health-risk behaviors among our nation's youth: United States, 1992. Vital Health Stat 192:1-51.

Adlard PA, King CE, Vickers JC. 2000. The effects of taxol on the central nervous system response to physical injury. Acta Neuropathol (Berl) 100(2):183-8.

Aihara N, Hall JJ, Pitts LH, Fukuda K, Noble LJ. 1995. Altered immunoexpression of microglia and macrophages after mild head injury. J Neurotrauma 12(1):53-63.

Airaksinen MS, Saarma M. 2002. The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci 3(5):383-94.

Aldskogius H, Kozlova EN. 1998. Central neuron-glial and glial-glial interactions following axon injury. Prog Neurobiol 55(1):1-26.

Alonso G. 2005. NG2 proteoglycan-expressing cells of the adult rat brain: possible involvement in the formation of glial scar astrocytes following stab wound. Glia 49(3):318-38.

Amor S, Puentes F, Baker D, van der Valk P. 2010. Inflammation in neurodegenerative diseases. Immunology 129(2):154-69.

Anderson KJ, Scheff SW, Miller KM, Roberts KN, Gilmer LK, Yang C, Shaw G. 2008. The phosphorylated axonal form of the neurofilament subunit NF-H (pNF-H) as a blood biomarker of traumatic brain injury. J Neurotrauma 25(9):1079-85.

Arai H, Emson PC, Mountjoy CQ, Carassco LH, Heizmann CW. 1987. Loss of parvalbumin-immunoreactive neurones from cortex in Alzheimer-type dementia. Brain Res 418(1):164-9.

Araque A, Navarrete M. 2010. Glial cells in neuronal network function. Philos Trans R Soc Lond B Biol Sci 365(1551):2375-81.

Argiro V, Bunge MB, Johnson MI. 1984. Correlation between growth form and movement and their dependence on neuronal age. J Neurosci 4(12):3051-62.

Arnold SE, Trojanowski JQ. 1996. Human fetal hippocampal development: II. The neuronal cytoskeleton. J Comp Neurol 367(2):293-307.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 8(9):963-70.

Ashe KH, Zahs KR. 2010. Probing the biology of Alzheimer's disease in mice. Neuron 66(5):631-45.

Ashery U, Penner R, Spira ME. 1996. Acceleration of membrane recycling by axotomy of cultured aplysia neurons. Neuron 16(3):641-51.

Bakshi A, Shimizu S, Keck CA, Cho S, LeBold DG, Morales D, Arenas E, Snyder EY, Watson DJ, McIntosh TK. 2006. Neural progenitor cells engineered to secrete GDNF show enhanced survival, neuronal differentiation and improve cognitive function following traumatic brain injury. Eur J Neurosci 23(8):2119-34.

Bandtlow CE, Schwab ME. 2000. NI-35/250/nogo-a: a neurite growth inhibitor restricting structural plasticity and regeneration of nerve fibres in the adult vertebrate CNS. Glia 29(2):175-81.

Banker G, Goslin K. 1988 Nov 10. Developments in neuronal cell culture. Nature 336(6195):185-6.

Baracskay KL, Kidd GJ, Miller RH, Trapp BD. 2007. NG2-positive cells generate A2B5-positive oligodendrocyte precursor cells. Glia 55(10):1001-10.

Bareyre FM, Kerschensteiner M, Raineteau O, Mettenleiter TC, Weinmann O, Schwab ME. 2004. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat Neurosci 7(3):269-77.

Barnabe-Heider F, Miller FD. 2003. Endogenously produced neurotrophins regulate survival and differentiation of cortical progenitors via distinct signaling pathways. J Neurosci 23(12):5149-60.

Barron KD. 1995. The microglial cell. A historical review. J Neurol Sci 134 Suppl:57-68.

Bayir H, Clark RS, Kochanek PM. 2003. Promising strategies to minimize secondary brain injury after head trauma. Crit Care Med 31(1 Suppl):S112-7.

Bear F.M., Connors B.W., M.A. P. 2001. Neuroscience Exploring The Brain.

Beaulieu JM, Jacomy H, Julien JP. 2000. Formation of intermediate filament protein aggregates with disparate effects in two transgenic mouse models lacking the neurofilament light subunit. J Neurosci 20(14):5321-8.

Belachew S, Chittajallu R, Aguirre AA, Yuan X, Kirby M, Anderson S, Gallo V. 2003. Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. J Cell Biol 161(1):169-86.

Benarroch EE. 2010. Glycogen metabolism: metabolic coupling between astrocytes and neurons. Neurology 74(11):919-23.

Benson DL, Mandell JW, Shaw G, Banker G. 1996. Compartmentation of alpha-internexin and neurofilament triplet proteins in cultured hippocampal neurons. J Neurocytol 25(3):181-96.

Bernhardt R, Matus A. 1984. Light and electron microscopic studies of the distribution of microtubule-associated protein 2 in rat brain: a difference between dendritic and axonal cytoskeletons. J Comp Neurol 226(2):203-21.

Berry M, Hall S, Shewan D, Cohen J. 1994. Axonal growth and its inhibition. Eye 8(Pt 2):245-54.

Beschorner R, Dietz K, Schauer N, Mittelbronn M, Schluesener HJ, Trautmann K, Meyermann R, Simon P. 2007. Expression of EAAT1 reflects a possible neuroprotective function of reactive astrocytes and activated microglia following human traumatic brain injury. Histol Histopathol 22(5):515-26.

Bielas SL, Gleeson JG. 2004. Cytoskeletal-associated proteins in the migration of cortical neurons. J Neurobiol 58(1):149-59.

Bignami A, Raju T, Dahl D. 1982. Localization of vimentin, the nonspecific intermediate filament protein, in embryonal glia and in early differentiating neurons. In vivo and in vitro immunofluorescence study of the rat embryo with vimentin and neurofilament antisera. Dev Biol 91(2):286-95.

Binder LI, Frankfurter A, Rebhun LI. 1985. The distribution of tau in the mammalian central nervous system. J Cell Biol 101(4):1371-8.

Black MM, Aletta JM, Greene LA. 1986. Regulation of microtubule composition and stability during nerve growth factor-promoted neurite outgrowth. J Cell Biol 103(2):545-57.

Blackmore M, Letourneau PC. 2006. Changes within maturing neurons limit axonal regeneration in the developing spinal cord. J Neurobiol 66(4):348-60.

Bradke F, Dotti CG. 1999 Mar 19. The role of local actin instability in axon formation. Science 283(5409):1931-4.

Bradl M, Lassmann H. 2010. Oligodendrocytes: biology and pathology. Acta Neuropathol 119(1):37-53.

Brandt R. 1998. Cytoskeletal mechanisms of axon outgrowth and pathfinding. Cell Tissue Res 292(2):181-189.

Braun H, Schafer K, Hollt V. 2002. BetaIII tubulin-expressing neurons reveal enhanced neurogenesis in hippocampal and cortical structures after a contusion trauma in rats. J Neurotrauma 19(8):975-83.

Brewer GJ. 1995. Serum-free B27-neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, spetum, cerebral cortex, cerebellum, and dentate gyrus. J Neurosci Res 42(5):674-683.

Brewer GJ. 1997. Isolation and culture of adult rat hippocampal neurons. J Neurosci Methods 71(2):143-55.

Brewer GJ, Torricelli JR, Evege EK, Price PJ. 1993. Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 35(5):567-76.

Brown CE, Boyd JD, Murphy TH. 2010. Longitudinal in vivo imaging reveals balanced and branch-specific remodeling of mature cortical pyramidal dendritic arbors after stroke. J Cereb Blood Flow Metab 30(4):783-791.

Brown CE, Li P, Boyd JD, Delaney KR, Murphy TH. 2007. Extensive turnover of dendritic spines and vascular remodeling in cortical tissues recovering from stroke. J Neurosci 27(15):4101-9.

Brown CE, Wong C, Murphy TH. 2008. Rapid morphologic plasticity of peri-infarct dendritic spines after focal ischemic stroke. Stroke 39(4):1286-91.

Buck KB, Zheng JQ. 2002 Nov 1. Growth cone turning induced by direct local modification of microtubule dynamics. J Neurosci 22(21):9358-67.

Buffo A, Rite I, Tripathi P, Lepier A, Colak D, Horn AP, Mori T, Gotz M. 2008. Origin and progeny of reactive gliosis: A source of multipotent cells in the injured brain. Proc Natl Acad Sci U S A 105(9):3581-6.

Burns KA, Murphy B, Danzer SC, Kuan CY. 2009. Developmental and post-injury cortical gliogenesis: a genetic fate-mapping study with Nestin-CreER mice. Glia 57(10):1115-29.

Bush PC, Prince DA, Miller KD. 1999. Increased pyramidal excitability and NMDA conductance can explain posttraumatic epileptogenesis without disinhibition: a model. J Neurophysiol 82(4):1748-58.

Cai D, Shen Y, De Bellard M, Tang S, Filbin MT. 1999. Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. Neuron 22(1):89-101.

Cantallops I, Routtenberg A. 1999. Activity-dependent regulation of axonal growth: posttranscriptional control of the GAP-43 gene by the NMDA receptor in developing hippocampus. J Neurobiol 41(2):208-20.

Carden MJ, Trojanowski JQ, Schlaepfer WW, Lee VM. 1987. Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. J Neurosci 7(11):3489-504.

Carlson NG, Rojas MA, Black JD, Redd JW, Hille J, Hill KE, Rose JW. 2009. Microglial inhibition of neuroprotection by antagonists of the EP1 prostaglandin E2 receptor. J Neuroinflammation 6:5.

Carmichael ST. 2003. Plasticity of cortical projections after stroke. Neuroscientist 9(1):64-75.

Carmichael ST. 2006. Cellular and molecular mechanisms of neural repair after stroke: making waves. Ann Neurol 59(5):735-42.

Caroni P. 1998. Neuro-regeneration: plasticity for repair and adaptation. Essays Biochem 33:53-64.

Carter J, Gragerov A, Konvicka K, Elder G, Weinstein H, Lazzarini RA. 1998. Neurofilament (NF) assembly; divergent characteristics of human and rodent NF-L subunits. J Biol Chem 273(9):5101-8.

Chen R, Cohen LG, Hallett M. 2002. Nervous system reorganization following injury. Neuroscience 111(4):761-73.

Chen S, Pickard JD, Harris NG. 2003a. Time course of cellular pathology after controlled cortical impact injury. Exp Neurol 182(1):87-102.

Chen XH, Iwata A, Nonaka M, Browne KD, Smith DH. 2003b. Neurogenesis and glial proliferation persist for at least one year in the subventricular zone following brain trauma in rats. J Neurotrauma 20(7):623-31.

Chilton JK. 2006. Molecular mechanisms of axon guidance. Dev Biol 292(1):13-24.

Chirumamilla S, Sun D, Bullock MR, Colello RJ. 2002. Traumatic brain injury induced cell proliferation in the adult mammalian central nervous system. J Neurotrauma 19(6):693-703.

Christman CW, Salvant JBJ, Walker SA, Povlishock JT. 1997. Characterization of a prolonged regenerative attempt by diffusely injured axons following traumatic brain injury in adult cat: a light and electron microscopic immunocytochemical study. Acta Neuropathol (Berl) 94(4):329-37.

Chuckowree JA, Dickson TC, Vickers JC. 2004. Intrinsic regenerative ability of mature CNS neurons. Neuroscientist 10(4):280-5.

Chuckowree JA, Vickers JC. 2003. Cytoskeletal and morphological alterations underlying axonal sprouting after localized transection of cortical neuron axons in vitro. J Neurosci 23(9):3715-3725.

Chung BG, Flanagan LA, Rhee SW, Schwartz PH, Lee AP, Monuki ES, Jeon NL. 2005. Human neural stem cell growth and differentiation in a gradient-generating microfluidic device. Lab Chip 5(4):401-6.

Chung RS, Vickers JC, Chuah MI, West AK. 2003. Metallothionein-IIA promotes initial neurite elongation and postinjury reactive neurite growth and facilitates healing after focal cortical brain injury. J Neurosci 23(8):3336-42.

Chung RS, Woodhouse A, Fung S, Dickson TC, West AK, Vickers JC, Chuah MI. 2004. Olfactory ensheathing cells promote neurite sprouting of injured axons in vitro by direct cellular contact and secretion of soluble factors. Cell Mol Life Sci 61(10):1238-45.

Clarke SR, Shetty AK, Bradley JL, Turner DA. 1994. Reactive astrocytes express the embryonic intermediate neurofilament nestin. Neuroreport 5(15):1885-8.

Cleveland DW, Monteiro MJ, Wong PC, Gill SR, Gearhart JD, Hoffman PN. 1991. Involvement of neurofilaments in the radial growth of axons. J Cell Sci Suppl 15:85-95.

Cochard P, Paulin D. 1984. Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo. J Neurosci 4(8):2080-94.

Cohen-Cory S, Fraser SE. 1995. Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. Nature 378(6553):192-6.

Cole RN, Hart GW. 2001 Dec. Cytosolic O-glycosylation is abundant in nerve terminals. J Neurochem 79(5):1080-9.

Compston A. 1995. Brain repair. J Intern Med 237(2):127-34.

Conover JC, Allen RL. 2002. The subventricular zone: new molecular and cellular developments. Cell Mol Life Sci 59(12):2128-35.

Corbin JG, Gaiano N, Juliano SL, Poluch S, Stancik E, Haydar TF. 2008. Regulation of neural progenitor cell development in the nervous system. J Neurochem 106(6):2272-87.

Cotman CW, Whittemore ER, Watt JA, Anderson AJ, Loo DT. 1994. Possible role of apoptosis in Alzheimer's disease. Ann N Y Acad Sci 747:36-49.

Cuadros MA, Navascues J. 1998. The origin and differentiation of microglial cells during development. Prog Neurobiol 56(2):173-89.

Cullheim S, Thams S. 2007. The microglial networks of the brain and their role in neuronal network plasticity after lesion. Brain Res Rev 55(1):89-96.

Darian-Smith C, Gilbert CD. 1994. Axonal sprouting accompanies functional reorganization in adult cat striate cortex. Nature 368(6473):737-40.

Davies SJ, Fitch MT, Memberg SP, Hall AK, Raisman G, Silver J. 1997. Regeneration of adult axons in white matter tracts of the central nervous system. Nature 390(6661):680-3.

Dawson MR, Levine JM, Reynolds R. 2000. NG2-expressing cells in the central nervous system: are they oligodendroglial progenitors? J Neurosci Res 61(5):471-9.

de Anda FC, Pollarolo G, Da Silva JS, Camoletto PG, Feiguin F, Dotti CG. 2005. Centrosome localization determines neuronal polarity. Nature 436(7051):704-8.

DeFelipe J, Jones EG, editors. 1991. Cajal's degneration and regeneration of the nervous system. New York: Oxford University Press.

Deitch JS, Banker GA. 1993. An electron microscopic analysis of hippocampal neurons developing in culture: early stages in the emergence of polarity. J Neurosci 13(10):4301-4315.

del Rio MR, DeFelipe J. 1997. Synaptic connections of calretinin-immunoreactive neurons in the human neocortex. J Neurosci 17(13):5143-54.

Deller T, Frotscher M. 1997. Lesion-induced plasticity of central neurons: sprouting of single fibres in the rat hippocampus after unilateral entorhinal cortex lesion. Prog Neurobiol 53(6):687-727.

Denk W, Strickler JH, Webb WW. 1990. Two-photon laser scanning fluorescence microscopy. Science 248(4951):73-6.

Dent EW, Gertler FB. 2003. Cytoskeletal dynamics and transport in growth cone motility and axon guidance. Neuron 40(2):209-27.

Dent EW, Kalil K. 2001. Axon branching requires interactions between dynamic microtubules and actin filaments. J Neurosci 21(24):9757-69.

Dent EW, Tang F, Kalil K. 2003. Axon guidance by growth cones and branches: common cytoskeletal and signaling mechanisms. Neuroscientist 9(5):343-53.

Di Filippo M, Tozzi A, Costa C, Belcastro V, Tantucci M, Picconi B, Calabresi P. 2008. Plasticity and repair in the post-ischemic brain. Neuropharmacology 55(3):353-62.

Di Giovanni S. 2009. Molecular targets for axon regeneration: focus on the intrinsic pathways. Expert Opin Ther Targets 13(12):1387-98.

Dickson BJ, Senti KA. 2002 Mar 19. Axon guidance: growth cones make an unexpected turn. Curr Biol 12(6):R218-20.

Dickson TC, Adlard PA, Vickers JC. 2000. Sequence of cellular changes following localized axotomy to cortical neurons in glia-free culture. J Neurotrauma 17(11):1095-1103.

Dickson TC, Chuckowree JA, Chuah MI, West AK, Vickers JC. 2005. alpha-Internexin immunoreactivity reflects variable neuronal vulnerability in Alzheimer's disease and supports the role of the beta-amyloid plaques in inducing neuronal injury. Neurobiol Dis 18(2):286-95.

Dickson TC, Chung RS, McCormack GH, Staal JA, Vickers JC. 2007. Acute reactive and regenerative changes in mature cortical axons following injury. Neuroreport 18(3):283-8.

Diefenbach TJ, Guthrie PB, Kater SB. 2000. Stimulus history alters behavioral responses of neuronal growth cones. J Neurosci 20(4):1484-1494.

DiLeonardi AM, Huh JW, Raghupathi R. 2009. Impaired axonal transport and neurofilament compaction occur in separate populations of injured axons following diffuse brain injury in the immature rat. Brain Res 1263:174-82.

Doetsch F. 2003. The glial identity of neural stem cells. Nat Neurosci 6(11):1127-34.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97(6):703-16.

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A. 1997. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci 17(13):5046-61.

Doetsch F, Hen R. 2005. Young and excitable: the function of new neurons in the adult mammalian brain. Curr Opin Neurobiol 15(1):121-8.

Dotti CG, Sullivan CA, Banker GA. 1988. The establishment of polarity by hippocampal neurons in culture. J Neurosci 8(4):1454-68.

Douen AG, Dong L, Vanance S, Munger R, Hogan MJ, Thompson CS, Hakim AM. 2004. Regulation of nestin expression after cortical ablation in adult rat brain. Brain Res 1008(2):139-46.

Douglas RJ, Martin KA. 2004. Neuronal circuits of the neocortex. Annu Rev Neurosci 27:419-51.

Dubois M, Strazielle C, Julien JP, Lalonde R. 2005. Mice with the deleted neurofilament of low molecular weight (Nefl) gene: 2. Effects on motor functions and spatial orientation. J Neurosci Res 80(6):751-8.

Duff D. 2001. Review article: altered states of consciousness, theories of recovery, and assessment following a severe traumatic brain injury. Axone 23(1):18-23.

Duggal N, Schmidt-Kastner R, Hakim AM. 1997. Nestin expression in reactive astrocytes following focal cerebral ischemia in rats. Brain Res 768(1-2):1-9.

Dunlop SA. 2008. Activity-dependent plasticity: implications for recovery after spinal cord injury. Trends Neurosci 31(8):410-8.

Edgar JM, Nave KA. 2009. The role of CNS glia in preserving axon function. Curr Opin Neurobiol 19(5):498-504.

Edgerton VR, Tillakaratne NJ, Bigbee AJ, de Leon RD, Roy RR. 2004. Plasticity of the spinal neural circuitry after injury. Annu Rev Neurosci 27:145-67.

Egeler-Peerdeman SM. 1993. Pathophysiology of Head Trauma. Neurosurgery 93:g.3-g.6.

Elston GN, Benavides-Piccione R, DeFelipe J. 2001. The pyramidal cell in cognition: a comparative study in human and monkey. J Neurosci 21(17):RC163.

Emery E, Aldana P, Bunge MB, Puckett W, Srinivasan A, Keane RW, Bethea J, Levi AD. 1998. Apoptosis after traumatic human spinal cord injury. J Neurosurg 89(6):911-20.

Emsley JG, Mitchell BD, Kempermann G, Macklis JD. 2005. Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. Prog Neurobiol 75(5):321-341.

Erez H, Spira ME. 2008. Local self-assembly mechanisms underlie the differential transformation of the proximal and distal cut axonal ends into functional and aberrant growth cones. J Comp Neurol 507(1):1019-30.

Escurat M, Djabali K, Gumpel M, Gros F, Portier MM. 1990. Differential expression of two neuronal intermediate-filament proteins, peripherin and the low-molecular-mass neurofilament protein (NF-L), during the development of the rat. J Neurosci 10(3):764-84.

Farkas O, Povlishock JT. 2007. Cellular and subcellular change evoked by diffuse traumatic brain injury: a complex web of change extending far beyond focal damage. Prog Brain Res 161:43-59.

Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV. 2004. Reactive astrocytes protect tissue and preserve function after spinal cord injury. J Neurosci 24(9):2143-55.

Fawcett J. 2009. Molecular control of brain plasticity and repair. Prog Brain Res 175:501-9.

Fawcett JW. 1992. Intrinsic neuronal determinants of regeneration. Trends Neurosci 15(1):5-8.

Fawcett JW. 1997. Astrocytic and neuronal factors affecting axon regeneration in the damaged central nervous system. Cell Tissue Res 290(2):371-7.

Fawcett JW. 2006. Novel strategies for protection and repair of the central nervous system. Clin Med 6(6):598-603.

Fawcett JW. 2006. Overcoming inhibition in the damaged spinal cord. J Neurotrauma 23(3-4):371-83.

Fawcett JW, Asher RA. 1999. The glial scar and central nervous system repair. Brain Res Bull 49(6):377-91.

Feldman JL, McCrimmon DR, Speck DF. 1984. Effect of synchronous activation of medullary inspiratory bulbo-spinal neurones on phrenic nerve discharge in cat. J Physiol 347:241-54.

Fellin T. 2009. Communication between neurons and astrocytes: relevance to the modulation of synaptic and network activity. J Neurochem 108(3):533-44.

Felten DL, Shetty AN. 2010. Netter's Atlas of Neuroscience.

Feng G, Mellor RH, Bernstein M, Keller-Peck C, Nguyen QT, Wallace M, Nerbonne JM, Lichtman JW, Sanes JR. 2000 Oct. Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron 28(1):41-51.

Filbin MT. 2003. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci 4(9):703-13.

Finnie JW, Blumbergs PC. 2002. Traumatic brain injury. Vet Pathol 39(6):679-89.

Fitzgerald J, Fawcett J. 2007. Repair in the central nervous system. J Bone Joint Surg Br 89(11):1413-20.

Floyd CL, Lyeth BG. 2007. Astroglia: important mediators of traumatic brain injury. Prog Brain Res 161:61-79.

Fonseca M, Soriano E. 1995. Calretinin-immunoreactive neurons in the normal human temporal cortex and in Alzheimer's disease. Brain Res 691(1-2):83-91.

Forscher P, Smith SJ. 1988. Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J Cell Biol 107(4):1505-16.

Frahm C, Haupt C, Witte OW. 2004. GABA neurons survive focal ischemic injury. Neuroscience 127(2):341-6.

Frisen J, Johansson CB, Lothian C, Lendahl U. 1998. Central nervous system stem cells in the embryo and adult. Cell Mol Life Sci 54(9):935-45.

Gallant PE. 1992 Mar. The direct effects of graded axonal compression on axoplasm and fast axoplasmic transport. J Neuropathol Exp Neurol 51(2):220-30.

Gallo G, Letourneau PC. 2004. Regulation of growth cone actin filaments by guidance cues. J Neurobiol 58(1):92-102.

Gallyas F, Pal J, Farkas O, Doczi T. 2006. The fate of axons subjected to traumatic ultrastructural (neurofilament) compaction: an electron-microscopic study. Acta Neuropathol 111(3):229-37.

Galtrey CM, Asher RA, Nothias F, Fawcett JW. 2007. Promoting plasticity in the spinal cord with chondroitinase improves functional recovery after peripheral nerve repair. Brain 130(Pt 4):926-39.

Gates MA, Thomas LB, Howard EM, Laywell ED, Sajin B, Faissner A, Gotz B, Silver J, Steindler DA. 1995. Cell and molecular analysis of the developing and adult mouse subventricular zone of the cerebral hemispheres. J Comp Neurol 361(2):249-66.

Gennarelli TA. 1996. The spectrum of traumatic axonal injury. Neuropathol Appl Neurobiol 22(6):509-13.

Genoud C, Quairiaux C, Steiner P, Hirling H, Welker E, Knott GW. 2006. Plasticity of astrocytic coverage and glutamate transporter expression in adult mouse cortex. PLoS Biol 4(11):e343.

Geraldo S, Gordon-Weeks PR. 2009. Cytoskeletal dynamics in growth-cone steering. J Cell Sci 122(Pt 20):3595-604.

Geraldo S, Khanzada UK, Parsons M, Chilton JK, Gordon-Weeks PR. 2008. Targeting of the F-actin-binding protein drebrin by the microtubule plus-tip protein EB3 is required for neuritogenesis. Nat Cell Biol 10(10):1181-9.

Goldberg JL, Barres BA. 2000. The relationship between neuronal survival and regeneration. Annu Rev Neurosci 23:579-612.

Goldberg JL, Klassen MP, Hua Y, Barres BA. 2002. Amacrine-signaled loss of intrinsic axon growth ability by retinal ganglion cells. Science 296(5574):1860-4.

Goldstein ME, Weiss SR, Lazzarini RA, Shneidman PS, Lees JF, Schlaepfer WW. 1988. mRNA levels of all three neurofilament proteins decline following nerve transection. Brain Res 427(3):287-91.

Gomes RA, Hampton C, El-Sabeawy F, Sabo SL, McAllister AK. 2006. The dynamic distribution of TrkB receptors before, during, and after synapse formation between cortical neurons. J Neurosci 26(44):11487-500.

Gordon-Weeks PR. 2004. Microtubules and growth cone function. J Neurobiol 58(1):70-83.

Goslin K, Schreyer DJ, Skene JH, Banker G. 1988. Development of neuronal polarity: GAP-43 distinguishes axonal from dendritic growth cones. Nature 336(6200):672-4.

Goslin K, Schreyer DJ, Skene JH, Banker G. 1990. Changes in the distribution of GAP-43 during the development of neuronal polarity. J Neurosci 10(2):588-602.

Grados-Munro EM, Fournier AE. 2003. Myelin-associated inhibitors of axon regeneration. J Neurosci Res 74(4):479-85.

Graham DI, McIntosh TK, Maxwell WL, Nicoll JA. 2000. Recent advances in neurotrauma. J Neuropathol Exp Neurol 59(8):641-51.

Grutzendler J, Kasthuri N, Gan WB. 2002. Long-term dendritic spine stability in the adult cortex. Nature 420(6917):812-6.

Gu W, Brannstrom T, Wester P. 2000. Cortical neurogenesis in adult rats after reversible photothrombotic stroke. J Cereb Blood Flow Metab 20(8):1166-73.

Gungabissoon RA, Bamburg JR. 2003. Regulation of Growth Cone Actin Dynamics by ADF/Cofilin. J Histochem Cytochem 51(4):411-20.

Haas MA, Vickers JC, Dickson TC, Chuckowree JA, Chung RS, Woodhouse A, Fung S, West AK, Chuah MI. 2004. Binding partners L1 cell adhesion molecule and the ezrin-radixin-moesin (ERM) proteins are involved in development and the regenerative response to injury of hippocampal and cortical neurons

Intrinsic regenerative ability of mature CNS neurons

Olfactory ensheathing cells promote neurite sprouting of injured axons in vitro by direct cellular contact and secretion of soluble factors. Eur J Neurosci 20(6):1436-44.

Haber M, Zhou L, Murai KK. 2006. Cooperative astrocyte and dendritic spine dynamics at hippocampal excitatory synapses. J Neurosci 26(35):8881-91.

Habgood MD, Bye N, Dziegielewska KM, Ek CJ, Lane MA, Potter A, Morganti-Kossmann C, Saunders NR. 2007. Changes in blood-brain barrier permeability to large and small molecules following traumatic brain injury in mice. Eur J Neurosci 25(1):231-8.

Hafidi A, Grumet M, Sanes DH. 2004. In vitro analysis of mechanisms underlying age-dependent failure of axon regeneration. J Comp Neurol 470(1):80-92.

Halassa MM, Fellin T, Takano H, Dong JH, Haydon PG. 2007. Synaptic islands defined by the territory of a single astrocyte. J Neurosci 27(24):6473-7.

Hall A, Lalli G. 2010. Rho and Ras GTPases in axon growth, guidance, and branching. Cold Spring Harb Perspect Biol 2(2):a001818.

Hall GF, Lee VM. 1995. Neurofilament sidearm proteolysis is a prominent early effect of axotomy in lamprey giant central neurons. J Comp Neurol 353(1):38-49.

Hall GF, Yao J. 2000 Neuronal morphology, axonal integrity, and axonal regeneration in situ are regulated by cytoskeletal phosphorylation in identified lamprey central neurons. Microsc Res Tech 48(1):32-46.

Halloran MC, Kalil K. 1994. Dynamic behaviors of growth cones extending in the corpus callosum of living cortical brain slices observed with video microscopy. J Neurosci 14(4):2161-2177.

Hamberger A, Huang YL, Zhu H, Bao F, Ding M, Blennow K, Olsson A, Hansson HA, Viano D, Haglid KG. 2003. Redistribution of neurofilaments and accumulation of beta-amyloid protein after brain injury by rotational acceleration of the head. J Neurotrauma 20(2):169-78.

Hammarlund M, Nix P, Hauth L, Jorgensen EM, Bastiani M. 2009. Axon regeneration requires a conserved MAP kinase pathway. Science 323(5915):802-6.

Harel NY, Strittmatter SM. 2006. Can regenerating axons recapitulate developmental guidance during recovery from spinal cord injury? Nat Rev Neurosci 7(8):603-16.

Hasan SJ, Keirstead HS, Muir GD, Steeves JD. 1993. Axonal regeneration contributes to repair of injured brainstem-spinal neurons in embryonic chick. J Neurosci 13(2):492-507.

Haydon PG. 2001. GLIA: listening and talking to the synapse. Nat Rev Neurosci 2(3):185-93.

Hely TA, Willshaw DJ. 1998. Short-term interactions between microtubules and actin filaments underlie long-term behaviour in neuronal growth cones. Proc Biol Sci 265(1407):1801-7.

Hendry SH, Jones EG, Emson PC, Lawson DE, Heizmann CW, Streit P. 1989. Two classes of cortical GABA neurons defined by differential calcium binding protein immunoreactivities. Exp Brain Res 76(2):467-72.

Hirrlinger J, Hulsmann S, Kirchhoff F. 2004. Astroglial processes show spontaneous motility at active synaptic terminals in situ. Eur J Neurosci 20(8):2235-9.

Hof PR, Glezer, II, Conde F, Flagg RA, Rubin MB, Nimchinsky EA, Vogt Weisenhorn DM. 1999. Cellular distribution of the calcium-binding proteins parvalbumin, calbindin, and calretinin in the neocortex of mammals: phylogenetic and developmental patterns. J Chem Neuroanat 16(2):77-116.

Hof PR, Morrison JH. 1991. Neocortical neuronal subpopulations labeled by a monoclonal antibody to calbindin exhibit differential vulnerability in Alzheimer's disease. Exp Neurol 111(3):293-301.

Hof PR, Nimchinsky EA. 1992. Regional distribution of neurofilament and calcium-binding proteins in the cingulate cortex of the macaque monkey. Cereb Cortex 2(6):456-67.

Hof PR, Nimchinsky EA, Celio MR, Bouras C, Morrison JH. 1993. Calretinin-immunoreactive neocortical interneurons are unaffected in Alzheimer's disease. Neurosci Lett 152(1-2):145-8.

Hoffman PN, Cleveland DW. 1988. Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: induction of a specific beta-tubulin isotype. Proc Natl Acad Sci U S A 85(12):4530-3.

Hoffman PN, Cleveland DW, Griffin JW, Landes PW, Cowan NJ, Price DL. 1987. Neurofilament gene expression: a major determinant of axonal caliber. Proc Natl Acad Sci U S A 84(10):3472-6.

Hoffman PN, Griffin JW, Gold BG, Price DL. 1985. Slowing of neurofilament transport and the radial growth of developing nerve fibers. J Neurosci 5(11):2920-9.

Hoffman PN, Griffin JW, Price DL. 1984. Control of axonal caliber by neurofilament transport. J Cell Biol 99(2):705-14.

Hoffman PN, Pollock SC, Striph GG. 1993 Jan. Altered gene expression after optic nerve transection: reduced neurofilament expression as a general response to axonal injury. Exp Neurol 119(1):32-6.

Hollenbeck PJ, Saxton WM. 2005. The axonal transport of mitochondria. J Cell Sci 118(Pt 23):5411-9.

Holmin S, Almqvist P, Lendahl U, Mathiesen T. 1997. Adult nestin-expressing subependymal cells differentiate to astrocytes in response to brain injury. Eur J Neurosci 9(1):65-75.

Holtmaat AJ, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW, Svoboda K. 2005. Transient and persistent dendritic spines in the neocortex in vivo. Neuron 45(2):279-91.

Honda T, Tabata H, Nakajima K. 2003. Cellular and molecular mechanisms of neuronal migration in neocortical development. Semin Cell Dev Biol 14(3):169-74.

Hou ST, Jiang SX, Smith RA. 2008. Permissive and repulsive cues and signalling pathways of axonal outgrowth and regeneration. Int Rev Cell Mol Biol 267:125-81.

Huang Z. 2009. Molecular regulation of neuronal migration during neocortical development. Mol Cell Neurosci 42(1):11-22.

Hung RJ, Yazdani U, Yoon J, Wu H, Yang T, Gupta N, Huang Z, van Berkel WJ, Terman JR. 2010. Mical links semaphorins to F-actin disassembly. Nature 463(7282):823-7.

Initiative TVN. 2009. The economic cost of spinal cord injury and traumatic brain injury in Australia. Access Economics.

Jacobs AJ, Swain GP, Snedeker JA, Pijak DS, Gladstone LJ, Selzer ME. 1997. Recovery of neurofilament expression selectively in regenerating reticulospinal neurons. J Neurosci 17(13):5206-20.

Jacobs KM, Graber KD, Kharazia VN, Parada I, Prince DA. 2000. Postlesional epilepsy: the ultimate brain plasticity. Epilepsia 41(Suppl 6):S153-61.

Jafari SS, Maxwell WL, Neilson M, Graham DI. 1997. Axonal cytoskeletal changes after non-disruptive axonal injury. J Neurocytol 26(4):207-21.

Jafari SS, Nielson M, Graham DI, Maxwell WL. 1998. Axonal cytoskeletal changes after nondisruptive axonal injury. II. Intermediate sized axons. J Neurotrauma 15(11):955-66.

Jellinger KA, Paulus W, Wrocklage C, Litvan I. 2001 Nov. Effects of closed traumatic brain injury and genetic factors on the development of Alzheimer's disease. Eur J Neurol 8(6):707-10.

Jiang XM, Zhao JX, Ohnishi A, Itakura C, Mizutani M, Yamamoto T, Murai Y, Ikeda M. 1996. Regeneration of myelinated fiber after crush injury is retarded in sciatic nerves of mutant Japanese quails deficient in neurofilaments. Acta Neuropathol (Berl) 92(5):467-72.

Jin K, Minami M, Lan JQ, Mao XO, Batteur S, Simon RP, Greenberg DA. 2001. Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. Proc Natl Acad Sci U S A 98(8):4710-5.

Jin X, Prince DA, Huguenard JR. 2006. Enhanced excitatory synaptic connectivity in layer v pyramidal neurons of chronically injured epileptogenic neocortex in rats. J Neurosci 26(18):4891-900.

Jones SL, Selzer ME, Gallo G. 2006. Developmental regulation of sensory axon regeneration in the absence of growth cones. J Neurobiol 66(14):1630-45.

Jones TA, Schallert T. 1992. Overgrowth and pruning of dendrites in adult rats recovering from neocortical damage. Brain Res 581(1):156-60.

Julien J-P, Grosveld F. 1991. Structure and Expression of Neurofilament Genes. The Neuronal Cytoskeleton:215-232.

Kaas JH, Krubitzer LA, Chino YM, Langston AL, Polley EH, Blair N. 1990. Reorganization of retinotopic cortical maps in adult mammals after lesions of the retina. Science 248(4952):229-31.

Kalil K. 1996. Growth cone behaviors during axon guidance in the developing cerebral cortex. Prog Brain Res 108:31-40.

Kalil K, Dent EW. 2005. Touch and go: guidance cues signal to the growth cone cytoskeleton. Curr Opin Neurobiol 15(5):521-6.

Kalil K, Szebenyi G, Dent EW. 2000. Common mechanisms underlying growth cone guidance and axon branching. J Neurobiol 44(2):145-158.

Kamber D, Erez H, Spira ME. 2009. Local calcium-dependent mechanisms determine whether a cut axonal end assembles a retarded endbulb or competent growth cone. Exp Neurol 219(1):112-25.

Kapfhammer JP. 1997. Axon sprouting in the spinal cord: growth promoting and growth inhibitory mechanisms. Anat Embryol (Berl) 196(6):417-26.

Kapfhammer JP, Schwab ME. 1994. Increased expression of the growth-associated protein GAP-43 in the myelin-free rat spinal cord. Eur J Neurosci 6(3):403-11.

Kaplan MP, Chin SS, Fliegner KH, Liem RK. 1990. Alpha-internexin, a novel neuronal intermediate filament protein, precedes the low molecular weight neurofilament protein (NF-L) in the developing rat brain. J Neurosci 10(8):2735-48.

Kawaguchi Y, Kubota Y. 1997. GABAergic cell subtypes and their synaptic connections in rat frontal cortex. Cereb Cortex 7(6):476-86.

Kelley BJ, Lifshitz J, Povlishock JT. 2007. Neuroinflammatory responses after experimental diffuse traumatic brain injury. J Neuropathol Exp Neurol 66(11):989-1001.

Kelly JP. 1981. Reactions of neurons to injury. In: Kandel ER, Schwartz JH, editors. Priniples of Neural Science. New York: Elsevier Noth-Holland. p 139-146.

Kernie SG, Erwin TM, Parada LF. 2001. Brain remodeling due to neuronal and astrocytic proliferation after controlled cortical injury in mice. J Neurosci Res 66(3):317-26.

Kerschensteiner M, Schwab ME, Lichtman JW, Misgeld T. 2005. In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. Nat Med 11(5):572-7.

Kimelberg HK. 1992. Astrocytic edema in CNS trauma. J Neurotrauma 9 Suppl 1:S71-81.

King AE, Chung RS, Vickers JC, Dickson TC. 2006. Localization of glutamate receptors in developing cortical neurons in culture and relationship to susceptibility to excitotoxicity. J Comp Neurol 498(2):277-294.

King CE, Adlard PA, Dickson TC, Vickers JC. 2000a. Neuronal response to physical injury and its relationship to the pathology of Alzheimer's disease. Clin Exp Pharmacol Physiol 27(7):548-52.

King CE, Canty AJ, Vickers JC. 2001. Alterations in neurofilaments associated with reactive brain changes and axonal sprouting following acute physical injury to the rat neocortex. Neuropathol Appl Neurobiol 27(2):115-26.

King CE, Dickson TC, Jacobs I, McCormack GH, Riederer BM, Vickers JC. 2000b. Acute CNS axonal injury models a subtype of dystrophic neurite in Alzheimer's disease. Alzheimer's Reports 3:31-40.

King CE, Jacobs I, Dickson TC, Vickers JC. 1997. Physical damage to rat cortical axons mimics early Alzheimer's neuronal pathology. Neuroreport 8(7):1663-5.

Knott GW, Quairiaux C, Genoud C, Welker E. 2002. Formation of dendritic spines with GABAergic synapses induced by whisker stimulation in adult mice. Neuron 34(2):265-73.

Kolb B, Gibb R. 1991. Environmental enrichment and cortical injury: behavioral and anatomical consequences of frontal cortex lesions. Cereb Cortex 1(2):189-98.

Korsching S. 1993. The neurotrophic factor concept: a reexamination. J Neurosci 13(7):2739-2748.

Kriegstein AR, Noctor SC. 2004. Patterns of neuronal migration in the embryonic cortex. Trends Neurosci 27(7):392-9.

Kronenberg G, Wang LP, Synowitz M, Gertz K, Katchanov J, Glass R, Harms C, Kempermann G, Kettenmann H, Endres M. 2005. Nestin-expressing cells divide and adopt a complex electrophysiologic phenotype after transient brain ischemia. J Cereb Blood Flow Metab 25(12):1613-24.

Krum JM, Rosenstein JM. 1999. Transient coexpression of nestin, GFAP, and vascular endothelial growth factor in mature reactive astroglia following neural grafting or brain wounds. Exp Neurol 160(2):348-60.

Kubo K, Nakajima K. 2003. Cell and molecular mechanisms that control cortical layer formation in the brain. Keio J Med 52(1):8-20.

Kuroda T, Nakamura H, Itoh K, Le WR, Yoshimura S, Takenaka K, Sakai N. 2002. Nestin immunoreactivity in local neurons of the adult rat striatum after remote cortical injury. J Chem Neuroanat 24(2):137-46.

Kwon BK, Borisoff JF, Tetzlaff W. 2002. Molecular targets for therapeutic intervention after spinal cord injury. Mol Interv 2(4):244-58.

Laird MD, Vender JR, Dhandapani KM. 2008. Opposing roles for reactive astrocytes following traumatic brain injury. Neurosignals 16(2-3):154-64.

Lamoureux P, Heidemann SR, Martzke NR, Miller KE. 2010. Growth and elongation within and along the axon. Dev Neurobiol 70(3):135-49.

Lariviere RC, Julien JP. 2004. Functions of intermediate filaments in neuronal development and disease. J Neurobiol 58(1):131-48.

Lee AC, Suter DM. 2008. Quantitative analysis of microtubule dynamics during adhesion-mediated growth cone guidance. Dev Neurobiol 68(12):1363-77.

Lee CY, Pappas GD, Kriho V, Huang BM, Yang HY. 2003. Proliferation of a subpopulation of reactive astrocytes following needle-insertion lesion in rat. Neurol Res 25(7):767-76.

Lee JK, Zheng B. 2008. Axon regeneration after spinal cord injury: insight from genetically modified mouse models. Restor Neurol Neurosci 26(2-3):175-82.

Lee MK, Cleveland DW. 1996. Neuronal intermediate filaments. Annu Rev Neurosci 19:187-217.

Lee MK, Xu Z, Wong PC, Cleveland DW. 1993. Neurofilaments are obligate heteropolymers in vivo. J Cell Biol 122(6):1337-50.

Lee WC, Chen JL, Huang H, Leslie JH, Amitai Y, So PT, Nedivi E. 2008. A dynamic zone defines interneuron remodeling in the adult neocortex. Proc Natl Acad Sci U S A 105(50):19968-73.

Lee WC, Huang H, Feng G, Sanes JR, Brown EN, So PT, Nedivi E. 2006. Dynamic remodeling of dendritic arbors in GABAergic interneurons of adult visual cortex. PLoS Biol 4(2):e29.

Lendahl U, Zimmerman LB, McKay RD. 1990. CNS stem cells express a new class of intermediate filament protein. Cell 60(4):585-95.

Leuba G, Kraftsik R, Saini K. 1998. Quantitative distribution of parvalbumin, calretinin, and calbindin D-28k immunoreactive neurons in the visual cortex of normal and Alzheimer cases. Exp Neurol 152(2):278-91.

Levine JM, Reynolds R, Fawcett JW. 2001. The oligodendrocyte precursor cell in health and disease. Trends Neurosci 24(1):39-47.

Li Y, Raisman G. 1995. Sprouts from cut corticospinal axons persist in the presence of astrocytic scarring in long-term lesions of the adult rat spinal cord. Exp Neurol 134(1):102-11.

Lie DC, Song H, Colamarino SA, Ming GL, Gage FH. 2004. Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annu Rev Pharmacol Toxicol 44:399-421.

Liem RK, Messing A. 2009. Dysfunctions of neuronal and glial intermediate filaments in disease. J Clin Invest 119(7):1814-24.

Limited AEP. 2009. The economic cost of spinal cord injury and traumatic brain injury in Australia. In: Initiative TVN, editor.

Lin H, Schlaepfer WW. 2006. Role of neurofilament aggregation in motor neuron disease. Ann Neurol 60(4):399-406.

Lin RC, Matesic DF, Marvin M, McKay RD, Brustle O. 1995. Re-expression of the intermediate filament nestin in reactive astrocytes. Neurobiol Dis 2(2):79-85.

Ling EA, Ng YK, Wu CH, Kaur C. 2001. Microglia: its development and role as a neuropathology sensor. Prog Brain Res 132:61-79.

Liou AK, Clark RS, Henshall DC, Yin XM, Chen J. 2003. To die or not to die for neurons in ischemia, traumatic brain injury and epilepsy: a review on the stress-activated signaling pathways and apoptotic pathways. Prog Neurobiol 69(2):103-42.

Lossinsky AS, Shivers RR. 2004. Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. Review. Histol Histopathol 19(2):535-64.

Lotan M, Schwartz M. 1994. Cross talk between the immune system and the nervous system in response to injury: implications for regeneration. Faseb J 8(13):1026-33.

Lowery LA, Van Vactor D. 2009. The trip of the tip: understanding the growth cone machinery. Nat Rev Mol Cell Biol 10(5):332-43.

Luebke JI, Weaver CM, Rocher AB, Rodriguez A, Crimins JL, Dickstein DL, Wearne SL, Hof PR. 2010. Dendritic vulnerability in neurodegenerative disease: insights from analyses of cortical pyramidal neurons in transgenic mouse models. Brain Struct Funct 214(2-3):181-99.

Lurie DI, Pijak DS, Selzer ME. 1994. Structure of reticulospinal axon growth cones and their cellular environment during regeneration in the lamprey spinal cord. J Comp Neurol 344(4):559-80.

Lynch JC. 2006. The cerebral cortex. In: Haines DU, editor. Fundamental Neuroscience for Basic Clinical Applications. Philadelphia: Churchill Livinstone. p 511-526.

Maas AI, Stocchetti N, Bullock R. 2008. Moderate and severe traumatic brain injury in adults. Lancet Neurol 7(8):728-41.

Macias M. 2008. Injury induced dendritic plasticity in the mature central nervous system. Acta Neurobiol Exp (Wars) 68(2):334-46.

Magavi SS, Leavitt BR, Macklis JD. 2000. Induction of neurogenesis in the neocortex of adult mice. Nature 405(6789):951-5.

Majewska A, Sur M. 2003. Motility of dendritic spines in visual cortex in vivo: changes during the critical period and effects of visual deprivation. Proc Natl Acad Sci U S A 100(26):16024-9.

Majewska AK, Newton JR, Sur M. 2006. Remodeling of synaptic structure in sensory cortical areas in vivo. J Neurosci 26(11):3021-9.

Marcos S, Moreau J, Backer S, Job D, Andrieux A, Bloch-Gallego E. 2009. Tubulin tyrosination is required for the proper organization and pathfinding of the growth cone. PLoS One 4(4):e5405.

Markram H, Toledo-Rodriguez M, Wang Y, Gupta A, Silberberg G, Wu C. 2004. Interneurons of the neocortical inhibitory system. Nat Rev Neurosci 5(10):793-807.

Marshall CA, Suzuki SO, Goldman JE. 2003. Gliogenic and neurogenic progenitors of the subventricular zone: who are they, where did they come from, and where are they going? Glia 43(1):52-61.

Martin LJ. 2001. Neuronal cell death in nervous system development, disease, and injury (Review). Int J Mol Med 7(5):455-78.

Masliah E, Mallory M, Ge N, Godson C, Saitoh T. 1993. Phorbol ester-induced neuritic alterations in the rat neocortex. Structural and immunocytochemical studies. Mol Chem Neuropathol 20(2):125-45.

Masliah E, Mallory M, Hansen L, Alford M, DeTeresa R, Terry R. 1993. An antibody against phosphorylated neurofilaments identifies a subset of damaged association axons in Alzheimer's disease. Am J Pathol 142(3):871-82.

Mason CA, Wang LC. 1997. Growth cone form is behavior-specific and, consequently, position-specific along the retinal axon pathway. J Neurosci 17(3):1086-100.

Maxwell WL, Follows R, Ashhurst DE, Berry M. 1990. The response of the cerebral hemisphere of the rat to injury. I. The mature rat. Philos Trans R Soc Lond B Biol Sci 328(1250):479-500.

Maxwell WL, Graham DI. 1997. Loss of axonal microtubules and neurofilaments after stretch-injury to guinea pig optic nerve fibers. J Neurotrauma 14(9):603-14.

McFarlane S. 2000. Attraction vs. repulsion: the growth cone decides. Biochem Cell Biol 78(5):563-8.

McGraw J, Hiebert GW, Steeves JD. 2001. Modulating astrogliosis after neurotrauma. J Neurosci Res 63(2):109-15.

McKinney RA, Debanne D, Gahwiler BH, Thompson SM. 1997. Lesion-induced axonal sprouting and hyperexcitability in the hippocampus in vitro: implications for the genesis of posttraumatic epilepsy. Nat Med 3(9):990-996.

McLean JR, Sanelli TR, Leystra-Lantz C, He BP, Strong MJ. 2005. Temporal profiles of neuronal degeneration, glial proliferation, and cell death in hNFL(+/+) and NFL(-/-) mice. Glia 52(1):59-69.

McTigue DM, Tripathi RB. 2008. The life, death, and replacement of oligodendrocytes in the adult CNS. J Neurochem 107(1):1-19.

McTigue DM, Wei P, Stokes BT. 2001. Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord. J Neurosci 21(10):3392-400.

Medina L, Abellan A. 2009. Development and evolution of the pallium. Semin Cell Dev Biol 20(6):698-711.

Mersiyanova IV, Perepelov AV, Polyakov AV, Sitnikov VF, Dadali EL, Oparin RB, Petrin AN, Evgrafov OV. 2000. A new variant of Charcot-Marie-Tooth disease type 2 is probably the result of a mutation in the neurofilament-light gene. Am J Hum Genet 67(1):37-46.

Michalczyk K, Ziman M. 2005. Nestin structure and predicted function in cellular cytoskeletal organisation. Histol Histopathol 20(2):665-71.

Mignone JL, Kukekov V, Chiang AS, Steindler D, Enikolopov G. 2004. Neural stem and progenitor cells in nestin-GFP transgenic mice. J Comp Neurol 469(3):311-24.

Molnar Z, Cheung AF. 2006. Towards the classification of subpopulations of layer V pyramidal projection neurons. Neurosci Res 55(2):105-15.

Morganti-Kossmann MC, Rancan M, Stahel PF, Kossmann T. 2002. Inflammatory response in acute traumatic brain injury: a double-edged sword. Curr Opin Crit Care 8(2):101-5.

Morrison B, 3rd, Saatman KE, Meaney DF, McIntosh TK. 1998. In vitro central nervous system models of mechanically induced trauma: a review. J Neurotrauma 15(11):911-28.

Muma NA, Hoffman PN, Slunt HH, Applegate MD, Lieberburg I, Price DL. 1990. Alterations in levels of mRNAs coding for neurofilament protein subunits during regeneration. Exp Neurol 107(3):230-5.

Myer DJ, Gurkoff GG, Lee SM, Hovda DA, Sofroniew MV. 2006. Essential protective roles of reactive astrocytes in traumatic brain injury. Brain 129(Pt 10):2761-72.

Nadarajah B, Brunstrom JE, Grutzendler J, Wong RO, Pearlman AL. 2001. Two modes of radial migration in early development of the cerebral cortex. Nat Neurosci 4(2):143-50.

Nadarajah B, Parnavelas JG. 2002. Modes of neuronal migration in the developing cerebral cortex. Nat Rev Neurosci 3(6):423-32.

Nagashima M, Dent EW, Shi XZ, Kalil K. 1999. Cortical neurite outgrowth and growth cone behaviors reveal developmentally regulated cues in spinal cord membranes. J Neurobiol 39(3):393-406.

Nakamura F, Kalb RG, Strittmatter SM. 2000 Aug. Molecular basis of semaphorin-mediated axon guidance. J Neurobiol 44(2):219-29.

Navascues J, Calvente R, Marin-Teva JL, Cuadros MA. 2000. Entry, dispersion and differentiation of microglia in the developing central nervous system. An Acad Bras Cienc 72(1):91-102.

Nedergaard M, Ransom B, Goldman SA. 2003. New roles for astrocytes: redefining the functional architecture of the brain. Trends Neurosci 26(10):523-30.

Neumann S, Bradke F, Tessier-Lavigne M, Basbaum AI. 2002. Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. Neuron 34(6):885-93.

Neumann S, Woolf CJ. 1999. Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. Neuron 23(1):83-91.

Nguyen MD, Shu T, Sanada K, Lariviere RC, Tseng HC, Park SK, Julien JP, Tsai LH. 2004. A NUDEL-dependent mechanism of neurofilament assembly regulates the integrity of CNS neurons. Nat Cell Biol 6(7):595-608.

Nimmerjahn A, Kirchhoff F, Helmchen F. 2005. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science 308(5726):1314-8.

Niwa H, Hayakawa K, Yamamoto M, Itoh T, Mitsuma T, Sobue G. 2002. Differential age-dependent trophic responses of nodose, sensory, and sympathetic neurons to neurotrophins and GDNF: potencies for neurite extension in explant culture. Neurochem Res 27(6):485-496.

Nixon RA. 1993. The regulation of neurofilament protein dynamics by phosphorylation: clues to neurofibrillary pathobiology. Brain Pathol 3(1):29-38.

Nixon RA, Shea TB. 1992. Dynamics of neuronal intermediate filaments: a developmental perspective. Cell Motil Cytoskeleton 22(2):81-91.

Nixon RA, Sihag RK. 1991. Neurofilament phosphorylation: a new look at regulation and function. Trends Neurosci 14(11):501-6.

Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR. 2004. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 7(2):136-44.

Nolte. 2009. The Human brain-an introduction to its functional anatomy. St Louis: Mosby.

Nudo RJ. 1999. Recovery after damage to motor cortical areas. Curr Opin Neurobiol 9(6):740-7.

Oehmichen M. 2004. Vitality and time course of wounds. Forensic Sci Int 144(2-3):221-31.

Okonkwo DO, Pettus EH, Moroi J, Povlishock JT. 1998. Alteration of the neurofilament sidearm and its relation to neurofilament compaction occurring with traumatic axonal injury. Brain Res 784(1-2):1-6.

Osterfield M, Kirschner MW, Flanagan JG. 2003. Graded positional information: interpretation for both fate and guidance. Cell 113(4):425-8.

Otsuka T, Kawaguchi Y. 2009. Cortical inhibitory cell types differentially form intralaminar and interlaminar subnetworks with excitatory neurons. J Neurosci 29(34):10533-40.

Pak CW, Flynn KC, Bamburg JR. 2008. Actin-binding proteins take the reins in growth cones. Nat Rev Neurosci 9(2):136-47.

Pappas IS, Parnavelas JG. 1998. Basic fibroblast growth factor promotes the generation and differentiation of calretinin neurons in the rat cerebral cortex in vitro. Eur J Neurosci 10(4):1436-45.

Parent JM. 2002. The role of seizure-induced neurogenesis in epileptogenesis and brain repair. Epilepsy Res 50(1-2):179-89.

Parent JM, Lowenstein DH. 2002. Seizure-induced neurogenesis: are more new neurons good for an adult brain? Prog Brain Res 135:121-31.

Parnavelas JG. 2000. The origin and migration of cortical neurones: new vistas. Trends Neurosci 23(3):126-31.

Parysek LM, Goldman RD. 1987. Characterization of intermediate filaments in PC12 cells. J Neurosci 7(3):781-91.

Pastor AM, Delgado-Garcia JM, Martinez-Guijarro FJ, Lopez-Garcia C, de La Cruz RR. 2000. Response of abducens internuclear neurons to axotomy in the adult cat. J Comp Neurol 427(3):370-90.

Perea G, Araque A. 2010. GLIA modulates synaptic transmission. Brain Res Rev 63(1-2):93-102.

Pettus EH, Christman CW, Giebel ML, Povlishock JT. 1994. Traumatically induced altered membrane permeability: its relationship to traumatically induced reactive axonal change. J Neurotrauma 11(5):507-22.

Pettus EH, Povlishock JT. 1996. Characterization of a distinct set of intra-axonal ultrastructural changes associated with traumatically induced alteration in axolemmal permeability. Brain Res 722(1-2):1-11.

Petzold A. 2005. Neurofilament phosphoforms: surrogate markers for axonal injury, degeneration and loss. J Neurol Sci 233(1-2):183-98.

Pfister KK. 1999. Cytoplasmic dynein and microtubule transport in the axon: the action connection. Mol Neurobiol 20(2-3):81-91.

Portera-Cailliau C, Pan DT, Yuste R. 2003. Activity-regulated dynamic behavior of early dendritic protrusions: evidence for different types of dendritic filopodia. J Neurosci 23(18):7129-42.

Portier MM, de Nechaud B, Gros F. 1983. Peripherin, a new member of the intermediate filament protein family. Dev Neurosci 6(6):335-44.

Posmantur RM, Kampfl A, Taft WC, Bhattacharjee M, Dixon CE, Bao J, Hayes RL. 1996. Diminished microtubule-associated protein 2 (MAP2) immunoreactivity following cortical impact brain injury. J Neurotrauma 13(3):125-37.

Posmantur RM, Newcomb JK, Kampfl A, Hayes RL. 2000. Light and confocal microscopic studies of evolutionary changes in neurofilament proteins following cortical impact injury in the rat. Exp Neurol 161(1):15-26.

Povlishock JT, Katz DI. 2005. Update of neuropathology and neurological recovery after traumatic brain injury. J Head Trauma Rehabil 20(1):76-94.

Povlishock JT, Marmarou A, McIntosh T, Trojanowski JQ, Moroi J. 1997. Impact acceleration injury in the rat: evidence for focal axolemmal change and related neurofilament sidearm alteration. J Neuropathol Exp Neurol 56(4):347-59.

Pryer NK, Walker RA, Skeen VP, Bourns BD, Soboeiro MF, Salmon ED. 1992. Brain microtubule-associated proteins modulate microtubule dynamic instability in vitro. Real-time observations using video microscopy. J Cell Sci 103 (Pt 4):965-76.

Raivich G, Bohatschek M, Kloss CU, Werner A, Jones LL, Kreutzberg GW. 1999. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain Res Brain Res Rev 30(1):77-105.

Rakic P. 2009. Evolution of the neocortex: a perspective from developmental biology. Nat Rev Neurosci 10(10):724-35.

Ramaswamy S, Goings GE, Soderstrom KE, Szele FG, Kozlowski DA. 2005. Cellular proliferation and migration following a controlled cortical impact in the mouse. Brain Res 1053(1-2):38-53.

Rao MV, Campbell J, Yuan A, Kumar A, Gotow T, Uchiyama Y, Nixon RA. 2003. The neurofilament middle molecular mass subunit carboxyl-terminal tail domains is essential for the radial growth and cytoskeletal architecture of axons but not for regulating neurofilament transport rate. J Cell Biol 163(5):1021-31.

Reeves TM, Phillips LL, Povlishock JT. 2005. Myelinated and unmyelinated axons of the corpus callosum differ in vulnerability and functional recovery following traumatic brain injury. Exp Neurol 196(1):126-37.

Reier PJ, Houle JD. 1988. The glial scar: its bearing on axonal elongation and transplantation approaches to CNS repair. Adv Neurol 47:87-138.

Reynolds BA, Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255(5052):1707-10.

Rezaie P, Dean A. 2002. Periventricular leukomalacia, inflammation and white matter lesions within the developing nervous system. Neuropathology 22(3):106-32.

Rezaie P, Trillo-Pazos G, Greenwood J, Everall IP, Male DK. 2002 Mar 10. Motility and ramification of human fetal microglia in culture: an investigation using time-lapse video microscopy and image analysis. Exp Cell Res 274(1):68-82.

Rhodes KE, Fawcett JW. 2004. Chondroitin sulphate proteoglycans: preventing plasticity or protecting the CNS? J Anat 204(1):33-48.

Rice AC, Khaldi A, Harvey HB, Salman NJ, White F, Fillmore H, Bullock MR. 2003. Proliferation and neuronal differentiation of mitotically active cells following traumatic brain injury. Exp Neurol 183(2):406-17.

Rice AC, Khaldi A, Harvey HB, Salman NJ, White F, Fillmore H, Bullock MR, Eriksson PS, Perfilieva E, Bjork-Eriksson T and others. 2003. Proliferation and neuronal differentiation of mitotically active cells following traumatic brain injury. Exp Neurol 183(2):406-17.

Richards LJ, Kilpatrick TJ, Bartlett PF. 1992. De novo generation of neuronal cells from the adult mouse brain. Proc Natl Acad Sci U S A 89(18):8591-5.

Ridet JL, Malhotra SK, Privat A, Gage FH. 1997. Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci 20(12):570-7.

Rivest S. 2009. Regulation of innate immune responses in the brain. Nat Rev Immunol 9(6):429-39.

Saatman KE, Graham DI, McIntosh TK. 1998. The neuronal cytoskeleton is at risk after mild and moderate brain injury. J Neurotrauma 15(12):1047-58.

Sabry JH, O'Connor TP, Evans L, Toroian-Raymond A, Kirschner M, Bentley D. 1991. Microtubule behavior during guidance of pioneer neuron growth cones in situ. J Cell Biol 115(2):381-95.

Sahly I, Khoutorsky A, Erez H, Prager-Khoutorsky M, Spira ME. 2006. On-line confocal imaging of the events leading to structural dedifferentiation of an axonal segment into a growth cone after axotomy. J Comp Neurol 494(5):705-20.

Salin P, Tseng GF, Hoffman S, Parada I, Prince DA. 1995. Axonal sprouting in layer V pyramidal neurons of chronically injured cerebral cortex. J Neurosci 15(12):8234-45.

Salman H, Ghosh P, Kernie SG. 2004. Subventricular zone neural stem cells remodel the brain following traumatic injury in adult mice. J Neurotrauma 21(3):283-92.

Sanchez-Camacho C, Bovolenta P. 2009. Emerging mechanisms in morphogen-mediated axon guidance. Bioessays 31(10):1013-25.

Sanes DH, Reh TA, Harris WA. 2006. Development of the Nervous system.

Sanford SD, Gatlin JC, Hokfelt T, Pfenninger KH. 2008. Growth cone responses to growth and chemotropic factors. Eur J Neurosci 28(2):268-78.

Santhakumar V, Ratzliff AD, Jeng J, Toth Z, Soltesz I. 2001. Long-term hyperexcitability in the hippocampus after experimental head trauma. Ann Neurol 50(6):708-17.

Saunders NR, Kitchener P, Knott GW, Nicholls JG, Potter A, Smith TJ. 1998. Development of walking, swimming and neuronal connections after complete spinal cord transection in the neonatal opossum, Monodelphis domestica. J Neurosci 18(1):339-55.

Schlaepfer WW. 1987. Neurofilaments: structure, metabolism and implications in disease. J Neuropathol Exp Neurol 46(2):117-29.

Schwab ME, Kapfhammer JP, Bandtlow CE. 1993. Inhibitors of neurite growth. Annu Rev Neurosci 16:565-95.

Schwegler G, Schwab ME, Kapfhammer JP. 1995. Increased collateral sprouting of primary afferents in the myelin-free spinal cord. J Neurosci 15(4):2756-67.

Scott D, Smith KE, O'Brien BJ, Angelides KJ. 1985. Characterization of mammalian neurofilament triplet proteins. Subunit stoichiometry and morphology of native and reconstituted filaments. J Biol Chem 260(19):10736-47.

Segal M, Barker JL. 1984. Rat hippocampal neurons in culture: voltage-clamp analysis of inhibitory synaptic connections. J Neurophysiol 52(3):469-87.

Selzer ME. 2003. Promotion of axonal regeneration in the injured CNS. Lancet Neurol 2(3):157-66.

Seress L, Gulyas AI, Ferrer I, Tunon T, Soriano E, Freund TF. 1993. Distribution, morphological features, and synaptic connections of parvalbumin- and calbindin D28k-immunoreactive neurons in the human hippocampal formation. J Comp Neurol 337(2):208-30.

Seth P, Koul N. 2008. Astrocyte, the star avatar: redefined. J Biosci 33(3):405-21.

Shaw G, Weber K. 1981. The distribution of the neurofilament triplet proteins within individual neurones. Exp Cell Res 136(1):119-25.

Shaw G, Weber K. 1982. Differential expression of neurofilament triplet proteins in brain development. Nature 298(5871):277-9.

Shea TB, Chan WK, Kushkuley J, Lee S. 2009. Organizational dynamics, functions, and pathobiological dysfunctions of neurofilaments. Results Probl Cell Differ 48:29-45.

Shimizu I, Oppenheim RW, O'Brien M, Shneiderman A. 1990. Anatomical and functional recovery following spinal cord transection in the chick embryo. J Neurobiol 21(6):918-37.

Silberberg G, Grillner S, Lebeau FE, Maex R, Markram H. 2005. Synaptic pathways in neural microcircuits. Trends Neurosci 28(10):541-51.

Silver J, Miller JH. 2004. Regeneration beyond the glial scar. Nat Rev Neurosci 5(2):146-56.

Simons M, Trajkovic K. 2006. Neuron-glia communication in the control of oligodendrocyte function and myelin biogenesis. J Cell Sci 119(Pt 21):4381-9.

Singleton RH, Povlishock JT. 2004. Identification and characterization of heterogeneous neuronal injury and death in regions of diffuse brain injury: evidence for multiple independent injury phenotypes. J Neurosci 24(14):3543-53.

Singleton RH, Zhu J, Stone JR, Povlishock JT. 2002. Traumatically induced axotomy adjacent to the soma does not result in acute neuronal death. J Neurosci 22(3):791-802.

Skaliora I, Adams R, Blakemore C. 2000. Morphology and growth patterns of developing thalamocortical axons. J Neurosci 20(10):3650-3662.

Smith DH, Chen XH, Nonaka M, Trojanowski JQ, Lee VM, Saatman KE, Leoni MJ, Xu BN, Wolf JA, Meaney DF. 1999 Sep. Accumulation of amyloid beta and tau and the formation of neurofilament inclusions following diffuse brain injury in the pig. J Neuropathol Exp Neurol 58(9):982-92.

Smith DH, Meaney DF, Shull WH. 2003. Diffuse axonal injury in head trauma. J Head Trauma Rehabil 18(4):307-16.

Sofroniew MV, Vinters HV. 2010. Astrocytes: biology and pathology. Acta Neuropathol 119(1):7-35.

Spira ME, Oren R, Dormann A, Gitler D. 2003. Critical calpain-dependent ultrastructural alterations underlie the transformation of an axonal segment into a growth cone after axotomy of cultured Aplysia neurons. J Comp Neurol 457(3):293-312.

Staal JA, Dickson TC, Chung RS, Vickers JC. 2009. Disruption of the ubiquitin proteasome system following axonal stretch injury accelerates progression to secondary axotomy. J Neurotrauma 26(5):781-8.

Steward O, Schauwecker PE, Guth L, Zhang Z, Fujiki M, Inman D, Wrathall J, Kempermann G, Gage FH, Saatman KE and others. 1999. Genetic approaches to neurotrauma research: opportunities and potential pitfalls of murine models. Exp Neurol 157(1):19-42.

Stone JR, Okonkwo DO, Dialo AO, Rubin DG, Mutlu LK, Povlishock JT, Helm GA. 2004. Impaired axonal transport and altered axolemmal permeability occur in distinct populations of damaged axons following traumatic brain injury. Exp Neurol 190(1):59-69.

Suter DM, Forscher P. 2000. Substrate-cytoskeletal coupling as a mechanism for the regulation of growth cone motility and guidance. J Neurobiol 44(2):97-113.

Swanwick CC, Harrison MB, Kapur J. 2004. Synaptic and extrasynaptic localization of brain-derived neurotrophic factor and the tyrosine kinase B receptor in cultured hippocampal neurons. J Comp Neurol 478(4):405-17.

Szebenyi G, Callaway JL, Dent EW, Kalil K. 1998. Interstitial branches develop from active regions of the axon demarcated by the primary growth cone during pausing behaviors. J Neurosci 18(19):7930-7940.

Tabata H, Nakajima K. 2003. Multipolar migration: the third mode of radial neuronal migration in the developing cerebral cortex. J Neurosci 23(31):9996-10001.

Tailby C, Wright LL, Metha AB, Calford MB. 2005. Activity-dependent maintenance and growth of dendrites in adult cortex. Proc Natl Acad Sci U S A 102(12):4631-6.

Takatsuru Y, Fukumoto D, Yoshitomo M, Nemoto T, Tsukada H, Nabekura J. 2009. Neuronal circuit remodeling in the contralateral cortical hemisphere during functional recovery from cerebral infarction. J Neurosci 29(32):10081-6.

Tan AM, Zhang W, Levine JM. 2005. NG2: a component of the glial scar that inhibits axon growth. J Anat 207(6):717-25.

Tanaka E, Sabry J. 1995. Making the connection: cytoskeletal rearrangements during growth cone guidance. Cell 83(2):171-6.

Tanaka EM, Kirschner MW. 1991. Microtubule behavior in the growth cones of living neurons during axon elongation. J Cell Biol 115(2):345-63.

Tao R, Aldskogius H. 1999. Glial cell responses, complement and apolipoprotein J expression following axon injury in the neonatal rat. J Neurocytol 28(7):559-70.

Tessier-Lavigne M, Goodman CS. 1996. The molecular biology of axon guidance. Science 274(5290):1123-1133.

Thaxton C, Bhat MA. 2009. Myelination and regional domain differentiation of the axon. Results Probl Cell Differ 48:1-28.

Thomas WE. 1992. Brain macrophages: evaluation of microglia and their functions. Brain Res Brain Res Rev 17(1):61-74.

Todd KJ, Serrano A, Lacaille JC, Robitaille R. 2006. Glial cells in synaptic plasticity. J Physiol Paris 99(2-3):75-83.

Tom VJ, Doller CM, Malouf AT, Silver J. 2004. Astrocyte-associated fibronectin is critical for axonal regeneration in adult white matter. J Neurosci 24(42):9282-90.

Tonchev AB, Yamashima T, Zhao L, Okano H. 2003. Differential proliferative response in the postischemic hippocampus, temporal cortex, and olfactory bulb of young adult macaque monkeys. Glia 42(3):209-24.

Toth C, Shim SY, Wang J, Jiang Y, Neumayer G, Belzil C, Liu WQ, Martinez J, Zochodne D, Nguyen MD. 2008. Ndel1 promotes axon regeneration via intermediate filaments. PLoS One 3(4):e2014.

Trachtenberg JT, Chen BE, Knott GW, Feng G, Sanes JR, Welker E, Svoboda K. 2002. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. Nature 420(6917):788-94.

Treherne JM, Woodward SK, Varga ZM, Ritchie JM, Nicholls JG. 1992. Restoration of conduction and growth of axons through injured spinal cord of neonatal opossum in culture. Proc Natl Acad Sci U S A 89(1):431-4.

Tucker KL, Meyer M, Barde YA. 2001. Neurotrophins are required for nerve growth during development. Nat Neurosci 4(1):29-37.

Tuszynski MH, Gage FH. 1995. Maintaining the neuronal phenotype after injury in the adult CNS. Neurotrophic factors, axonal growth substrates, and gene therapy. Mol Neurobiol 10(2-3):151-67.

Twiss JL, van Minnen J. 2006. New insights into neuronal regeneration: the role of axonal protein synthesis in pathfinding and axonal extension. J Neurotrauma 23(3-4):295-308.

Vanek P, Thallmair M, Schwab ME, Kapfhammer JP. 1998. Increased lesion-induced sprouting of corticospinal fibres in the myelin-free rat spinal cord. Eur J Neurosci 10(1):45-56.

Varga ZM, Bandtlow CE, Erulkar SD, Schwab ME, Nicholls JG. 1995. The critical period for repair of CNS of neonatal opossum (Monodelphis domestica) in culture: correlation with development of glial cells, myelin and growth-inhibitory molecules. Eur J Neurosci 7(10):2119-29.

Vickers JC, Dickson TC, Adlard PA, Saunders HL, King CE, McCormack G. 2000. The cause of neuronal degeneration in Alzheimer's disease. Prog Neurobiol 60(2):139-65.

Voelker CC, Garin N, Taylor JS, Gahwiler BH, Hornung JP, Molnar Z. 2004. Selective neurofilament (SMI-32, FNP-7 and N200) expression in subpopulations of layer V pyramidal neurons in vivo and in vitro. Cereb Cortex 14(11):1276-86.

Walker KL, Yoo HK, Undamatla J, Szaro BG. 2001. Loss of neurofilaments alters axonal growth dynamics. J Neurosci 21(24):9655-66.

Waterman-Storer CM, Karki SB, Kuznetsov SA, Tabb JS, Weiss DG, Langford GM, Holzbaur EL. 1997 Oct 28. The interaction between cytoplasmic dynein and dynactin is required for fast axonal transport. Proc Natl Acad Sci U S A 94(22):12180-5.

Watkins TA, Barres BA. 2002. Nerve regeneration: regrowth stumped by shared receptor. Curr Biol 12(19):R654-6.

Waxman SG, Ritchie JM. 1993. Molecular dissection of the myelinated axon. Ann Neurol 33(2):121-36.

Wen Z, Guirland C, Ming GL, Zheng JQ. 2004. A CaMKII/calcineurin switch controls the direction of Ca(2+)-dependent growth cone guidance. Neuron 43(6):835-46.

Werner C, Engelhard K. 2007. Pathophysiology of traumatic brain injury. Br J Anaesth 99(1):4-9.

White RE, Jakeman LB. 2008. Don't fence me in: harnessing the beneficial roles of astrocytes for spinal cord repair. Restor Neurol Neurosci 26(2-3):197-214.

Willard M, Simon C. 1983. Modulations of neurofilament axonal transport during the development of rabbit retinal ganglion cells. Cell 35(2 Pt 1):551-9.

Willmore LJ. 1995. Post-traumatic epilepsy: mechanisms and prevention. Psychiatry Clin Neurosci 49(3):S171-3.

Xu X, Callaway EM. 2009. Laminar specificity of functional input to distinct types of inhibitory cortical neurons. J Neurosci 29(1):70-85.

Xu X, Roby KD, Callaway EM. 2006. Immunochemical characterization of inhibitory mouse cortical neurons: three chemically distinct classes of inhibitory cells. J Comp Neurol 518(3):389-404.

Yaghmai A, Povlishock J. 1992. Traumatically induced reactive change as visualized through the use of monoclonal antibodies targeted to neurofilament subunits. J Neuropathol Exp Neurol 51(2):158-76.

Yamahachi H, Marik SA, McManus JN, Denk W, Gilbert CD. 2009. Rapid axonal sprouting and pruning accompany functional reorganization in primary visual cortex. Neuron 64(5):719-29.

Yiu G, He Z. 2006. Glial inhibition of CNS axon regeneration. Nat Rev Neurosci 7(8):617-27.

Yu TS, Zhang G, Liebl DJ, Kernie SG. 2008. Traumatic brain injury-induced hippocampal neurogenesis requires activation of early nestin-expressing progenitors. J Neurosci 28(48):12901-12.

Yuan A, Rao MV, Kumar A, Julien JP, Nixon RA. 2003. Neurofilament transport in vivo minimally requires hetero-oligomer formation. J Neurosci 23(28):9452-8.

Yuan A, Rao MV, Sasaki T, Chen Y, Kumar A, Veeranna, Liem RK, Eyer J, Peterson AC, Julien JP and others. 2006. Alpha-internexin is structurally and functionally associated with the neurofilament triplet proteins in the mature CNS. J Neurosci 26(39):10006-19.

Yukawa K, Tanaka T, Yoshida K, Takeuchi N, Ito T, Takamatsu H, Kikutani H, Kumanogoh A. 2010. Sema4A induces cell morphological changes through B-type plexin-mediated signaling. Int J Mol Med 25(2):225-30.

Zhang G, Jin LQ, Sul JY, Haydon PG, Selzer ME. 2005. Live imaging of regenerating lamprey spinal axons. Neurorehabil Neural Repair 19(1):46-57.

Zhang SX, Underwood M, Landfield A, Huang FF, Gison S, Geddes JW. 2000. Cytoskeletal disruption following contusion injury to the rat spinal cord. J Neuropathol Exp Neurol 59(4):287-96.

Zhang W, Benson DL. 2001 Jul 15. Stages of synapse development defined by dependence on F-actin. J Neurosci 21(14):5169-81.

Zhang Z, Casey DM, Julien JP, Xu Z. 2002. Normal dendritic arborization in spinal motoneurons requires neurofilament subunit L. J Comp Neurol 450(2):144-52.

Zhou FQ, Cohan CS. 2004. How actin filaments and microtubules steer growth cones to their targets. J Neurobiol 58(1):84-91.

Zhou FQ, Snider WD. 2006. Intracellular control of developmental and regenerative axon growth. Philos Trans R Soc Lond B Biol Sci 361(1473):1575-92.

Zhou L, Shine HD. 2003. Neurotrophic factors expressed in both cortex and spinal cord induce axonal plasticity after spinal cord injury. J Neurosci Res 74(2):221-6.

Zhu Q, Couillard-Despres S, Julien JP. 1997. Delayed maturation of regenerating myelinated axons in mice lacking neurofilaments. Exp Neurol 148(1):299-316.

Ziebell JM, Morganti-Kossmann MC. 2010. Involvement of pro- and anti-inflammatory cytokines and chemokines in the pathophysiology of traumatic brain injury. Neurotherapeutics 7(1):22-30.

9 APPENDIX

9.1 SOLUTIONS

9.1.1 General solutions

0.01M PBS, pH 7.4

100mL	9% NaCl (90g of NaCl (Sigma, USA) per 1L Milli-Q® water)
40mL	Na_2HPO_4 (BDH) (28.4g per 1L Milli-Q® water)
10mL	$NaH_2PO_4.2H_2O$ (Sigma) (31.2g per 1L Milli-Q® water)
850mL	Milli-Q® water

9.1.2 Cell culture solutions

0.001% poly-l-lysine

450mL	sterile 0.01M PBS
50mL	0.01% poly-l-lysine (Sigma, USA)

10mM HEPES buffer

1.19g	HEPES (BDH, USA)
500mL	0.01M PBS

Combine and heat sterilise

Imaging buffer

15.5mL	4M NaCl (23.4g per 100mL Milli-Q® water)
2.5mL	2M KCl (Sigma; 7.46g per 50mL Milli-Q® water)
50mL	2M $CaCl_2$ (BDH; 14.7g per 50mL Milli-Q® water
250mL	2M $MgCl_2$ (Sigma; 20.33g per 50mL Milli-Q® water)
7.5mL	2M Dextrose (D-glucose) (Sigma; 18.02g per 50mL Milli-Q® water)
6.25mL	2M HEPES (26.03g per 50mL Milli-Q® water)

Adjust to pH 7.3 with 2M NaOH, make up to 500mL with Milli-Q® water, filter sterilise and store at 4°C.

9.1.3 Immunocytochemistry and immunohistochemistry solutions

4% Paraformaldehyde (PFA)

40g	PFA (Sigma, USA)
40g	Sucrose (Sigma, USA)
100mL	9% NaCl
400mL	Na_2HPO_4
500mL	$NaH_2PO_4.2H_2O$

Heat while stirring in a fume hood.

0.3% Triton/PBS

600µL	Triton X (Fluka, Switzerland)
200mL	0.01M PBS

9.1.4 Immunoblotting solutions

Bis/Acrylamide

29.2g	acrylamide (Sigma, USA)
0.8g	N'N'-bis-methylene-acrylamide (Sigma, USA)

Make up to 100mL with Milli-Q® water, filter through Whatman filter paper and store at 4°C, in the dark

1.5M Tris-HCl, pH 8.8

27.23g	Tris base (Bio-Rad, USA)
~80mL	Milli-Q® water

Adjust to pH 8.8 with 10M HCl and make up to 150mL with Milli-Q® water. Store at 4°C.

0.5M Tris-HCl, pH 6.8

6g	Tris base

~60mL Milli-Q® water

Adjust to pH 6.8 with 10M HCl and make up to 100mL with Milli-Q® water. Store at 4°C.

10% Sodium Dodecyl Sulfate (SDS)

10g SDS (BDH, USA)

Dissolve in 90mL of Milli-Q® water with gentle stirring and adjust to 100mL with Milli-Q®. Store at room temperature.

5X Running Buffer, pH 8.3

9g Tris base

43.2g Glycine (Bio-Rad, USA)

3g SDS

Combine and add 600mL of Milli-Q® water. Store at 4°C. Prior to use combine 100mL Running Buffer with 400mL Milli-Q® water.

Transfer Buffer

3.03g Tris base

14.4g Glycine

200mL Methanol (Sigma, USA)

Combine reagents in a fume cabinet and make up to 1L with 800mL of Milli-Q® water. Store at room temperature.

Tris buffered saline (TBS)

4.84g Tris base

58.4g NaCl

1.5L Milli-Q®

Adjust to pH 7.5 with 10M HCl and make up to 2L with Milli-Q® water.

Tris buffered saline with Tween (TTBS)

1.1L TBS

550uL Tween-20 (Bio-Rad, USA)

TTBS/5%FCS/5% skim milk powder

100mL TTBS

5mL FCS (Gibco, USA)

5g skim milk powder

Store at 4°C for a limited time.

12% Separating gel

3.35mL Milli-Q® water

2.5mL 1.5M Tris-HCl, pH 8.8

100µL 10% SDS

4mL Bis/Acrylamide

Add quickly immediately prior to pouring

50µL 10% APS (Bio-Rad, USA)

5µL TEMED (Bio-Rad, USA)

Combine ingredients in the listed order and pour gel immediately. Allow to polymerise for 45 minutes.

7.5% Separating gel

4.85mL Milli-Q® water

2.5mL 1.5M Tris-HCl, pH 8.8

100µL 10% SDS

2.5mL Bis/Acrylamide

Add quickly immediately prior to pouring

50µL 10% APS

5µL TEMED

Combine ingredients in the listed order and pour gel immediately. Allow to polymerise for 45 minutes.

4% Stacking gel

6.1mL Milli-Q® water

2.5mL 0.5M Tris-HCl, pH 6.8

100µL	10% SDS
1.33mL	Bis/Acrylamide
50µL	10% APS
10µL	TEMED

Coomassie stain

1g	Coomassie blue R-250 (Bio-Rad, USA)
40mL	methanol, 10%
10mL	acetic acid (Spectrum, USA)

Coomassie destain

40mL	methanol, 10%
10mL	acetic acid
50mL	Milli-Q® water

2X Sample buffer

2.5mL	0.5M Tris HCl pH6.8
2mL	glycerol (Sigma)
200µL	2-β mercaptoethanol (Serva)
2mL	10% SDS (in water)

Make up to 10mL with Milli-Q® water, add a small amount of bromophenol blue, aliquot and freeze

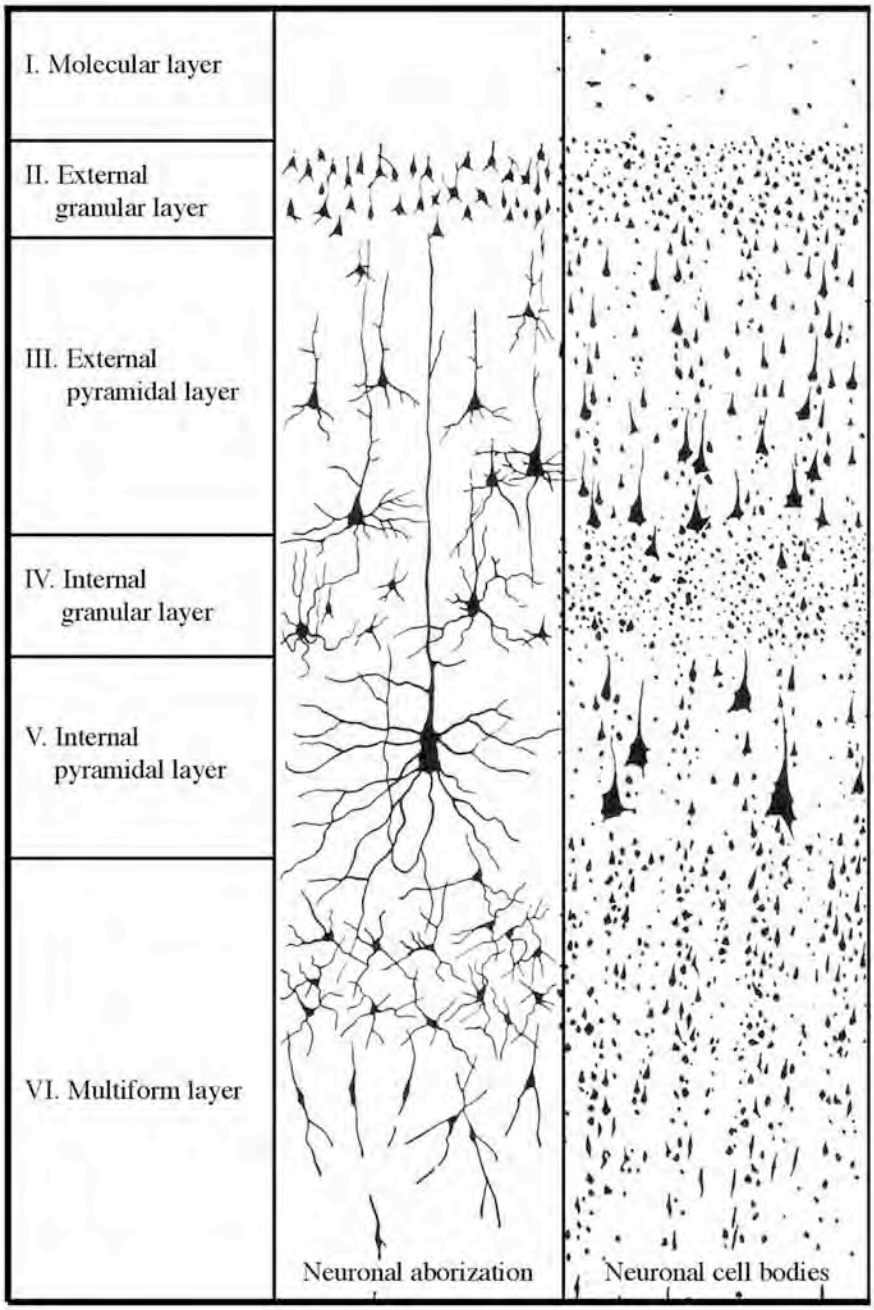


Figure 1.1. Laminar structure of the neocortex. Schematic representation of the six layers of the neocortex that form during CNS development demonstrating neuronal aborisation and packing density of cell bodies. The names of each layer reflect the cell types that predominantly reside in that layer. Precursor neurons must undergo a highly co-ordinated migratory journey from their birthplace in order to take up these specific positions (Adapted from Nolte, 2009).

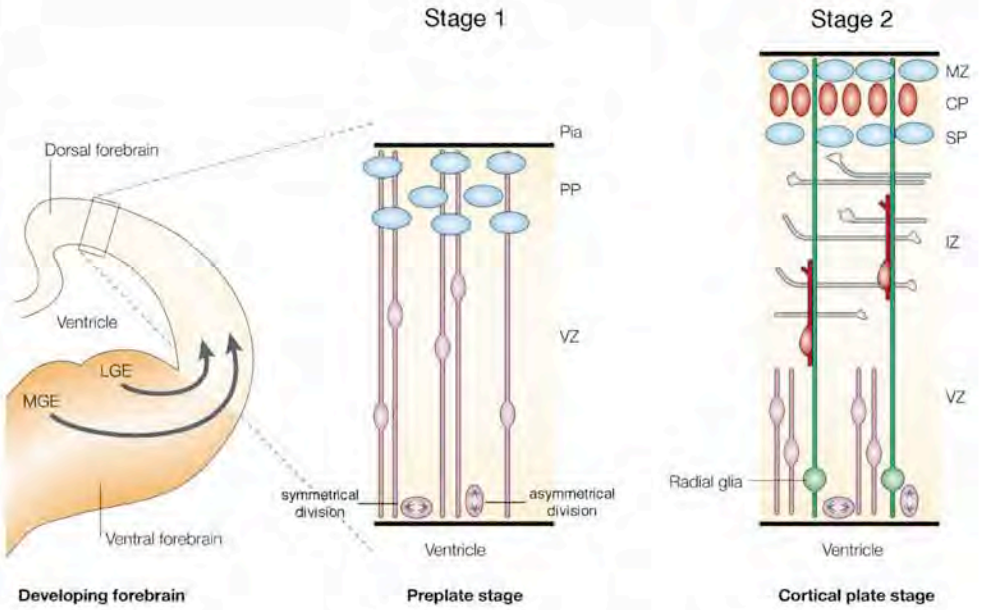


Figure 1.2 Development of the mammalian neocortex. During embryonic development the dorsal forebrain creates the neocortex. Neuronal progenitors destined to differentiate into pyramidal neurons are derived from the ventricular zone (VZ), whereas progenitors destined to differentiate into cortical interneurons are predominantly derived from the medial and lateral ganglionic eminences (MGE and LGE, respectively) in the ventral forebrain.

The neocortex develops in two distinct stages. Firstly post-mitotic neurons migrate toward the margin of the cerebral wall to form the primordial plexiform layer, or preplate (A). Secondly new born neurons migrate into the preplate and as they accumulate, form a new series of layers called the cortical plate, forming the superficialmarginal zone (layer I) and a deeper layer, the subplate (layers II-VI); The later born post-mitotic neurons that form layers II-VI of the cortical plate are arranged in an 'inside-out' sequence (B) (Adapted Nadarajahand Parnavelas 2002).

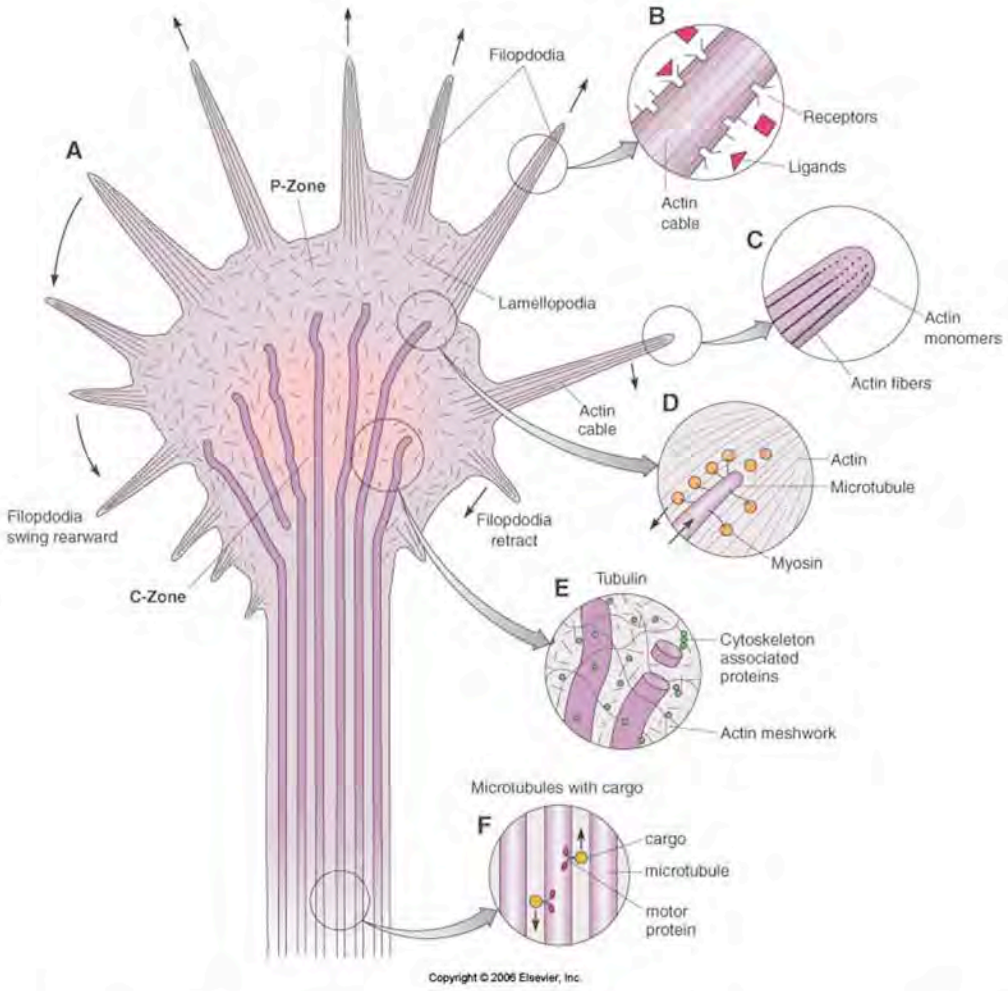


Figure 1.3. The neuronal growth cone. Diagrammatic representation of the growth cone. The peripheral (P) domain consists of long slender, highly dynamic filopdial protrusions, which contain F-actin, longitudally arranged in bundles, but very few microtubules. Lamellipodia are broad, fan shaped structures which consist of crosslinked F-actin, and microtubules. The central (C-zone) domain contains F-actin and microtubules. The guidance and growth of growth cones is determined by the interactions of filapodia with the extrinsic environment (A) through transmembrane receptors (B). The surface of the filopodium is rich in transmembrane proteins which act as receptors for cell adhesion molecules. F-actin forms rod-like parallel bundles within the filopodium (C). Growth cones extension is enabled by F-actin and microtubule interactions within the peripheral zone (D). Within the axonal shaft, microtubules form a dense parallel array that upon entering the growth cone splays apart; the microtubules contained within the central zone are in a state of dynamic instability, which means they are constantly undergoing rapid periods of growth and shrinkage (E). Within the axon, microtubules also participate in the active process of axonal transport (F) (Adapted from Sanes et al., 2006).

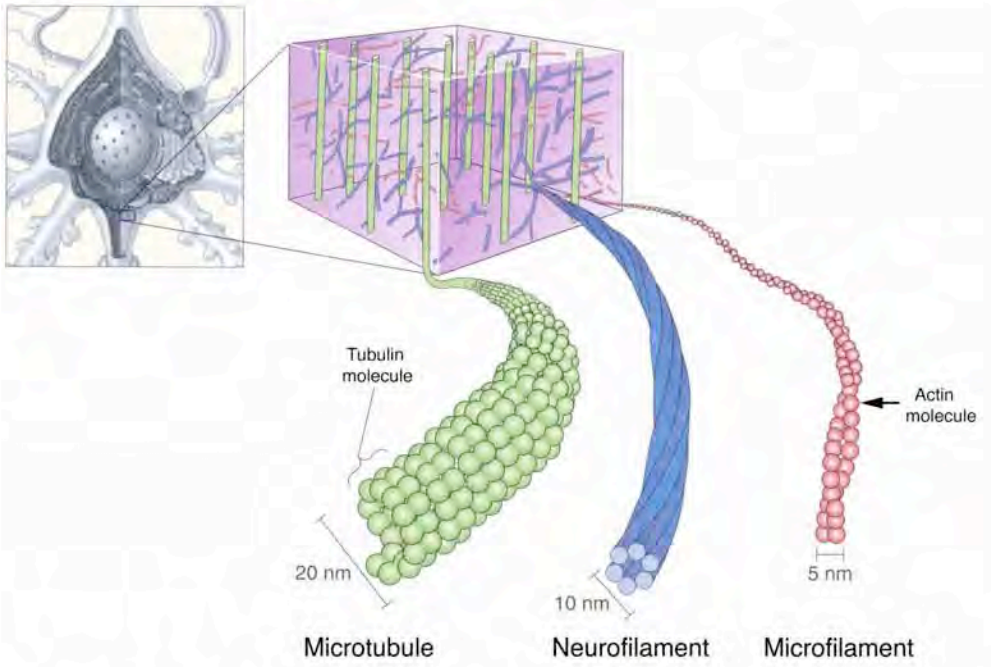


Figure 1.4 Diagrammatic representation of the neuronal cytoskeleton. The neuronal cytoskeleton is composed of microfilaments, neuronal intermediate filaments (neurofilaments) and microtubules. Microtubules are tube-like structures, composed of tubulin molecules. Neurofilaments, unlike microfilaments and microtubules, are composed of subunits with a filamentous structure. Microfilaments are the smallest diameter cytoskeletal component and are composed of helically intertwined strands of polymerised actin molecules (Adapted from Bear et al., 2001).

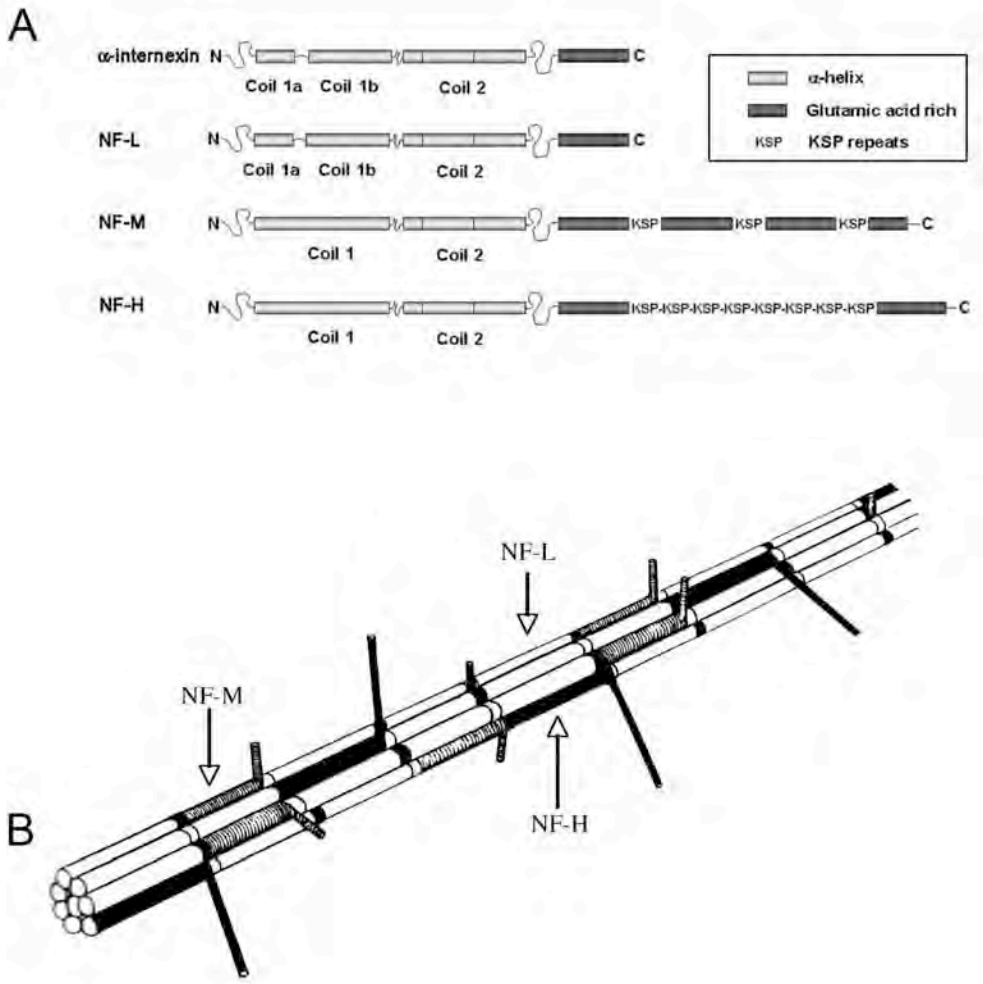


Figure 1.5 Type IV neuronal intermediate filament proteins. Diagrammatic representation of type IV neuronal intermediate filament proteins (A). Type IV neuronal intermediate filament proteins comprise the main intermediate filament component of the cytoskeleton of most mature CNS neurons and include alpha-internexin and the neurofilament triplet proteins, NF-L, NF-M and NF-H, commonly referred to as neurofilaments.Neuronal intermediate filaments have a distinct filamentous structure (B). Neurofilament triplet proteins are polymers of NF-L, NF-M and NF-H subunits. NF-M and NF-H possess side arms (carboxy terminal tail domains) that protrude from the core filament, vary in length between sub-units and undergo phosphorylation to increase inter filament spacing. (Adapted from Lariviere and Julien, 2004).

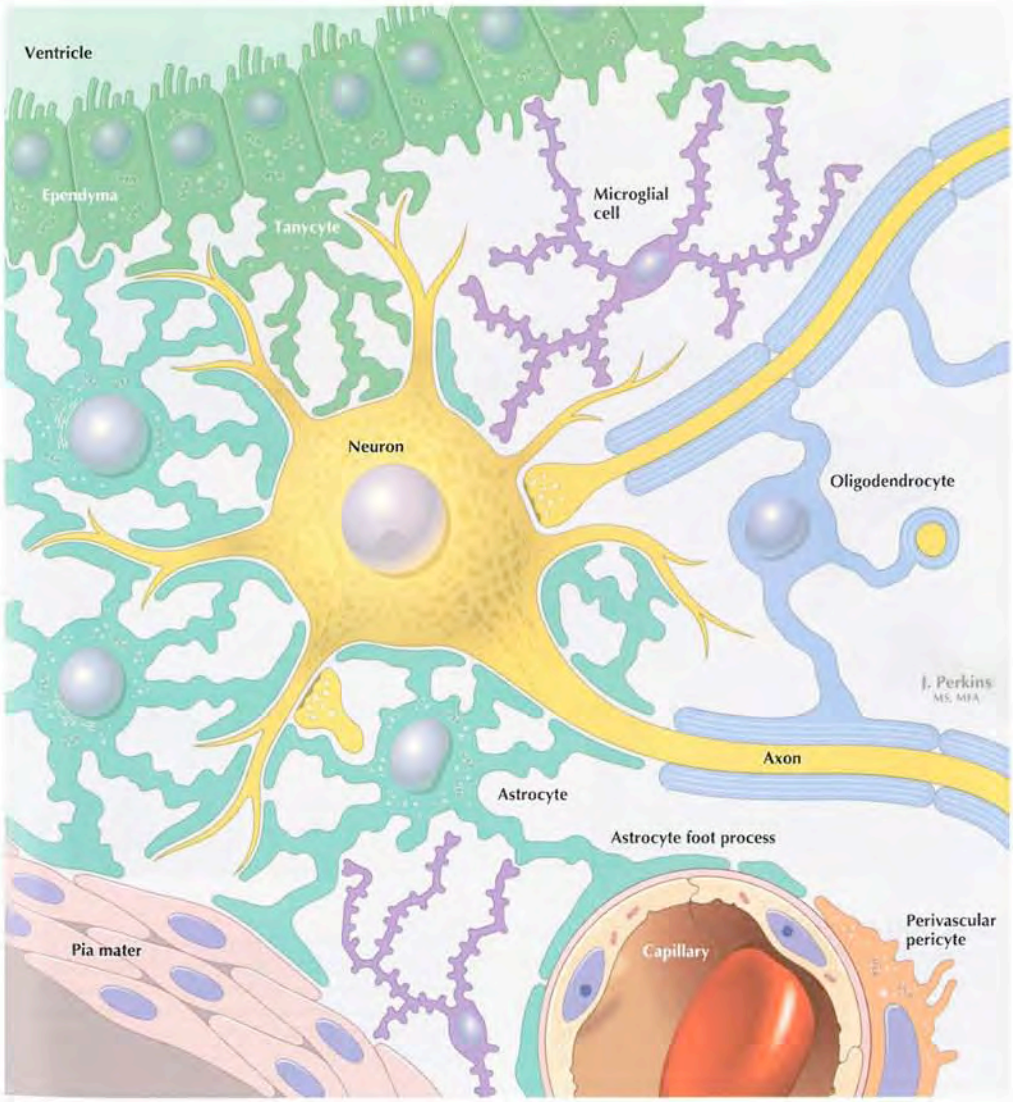


Figure 1.6. Glial cells of the CNS. Schematic representation of all the different types of glial cells and their interactions. Astrocytes are stellate-shaped cells which play vital roles in formation of the blood brain barrier, maintaining local ion concentrations and pH homeostasis in the extracellular space, assisting neurons in the turnover of nutrients and metabolic by-products and neurotransmitters (glutamate), regulate synapse development and modulate synaptic strength. Oligodendrocytes are the myelinating cells of the CNS, their arborisations forming myelin sheaths around the axons belonging to CNS neurons, this sheath broken up along the axon by nodes of Ranvier. Microglial cells are the primary immune cell type of the CNS (Taken from Felten and Shetty, 2010).

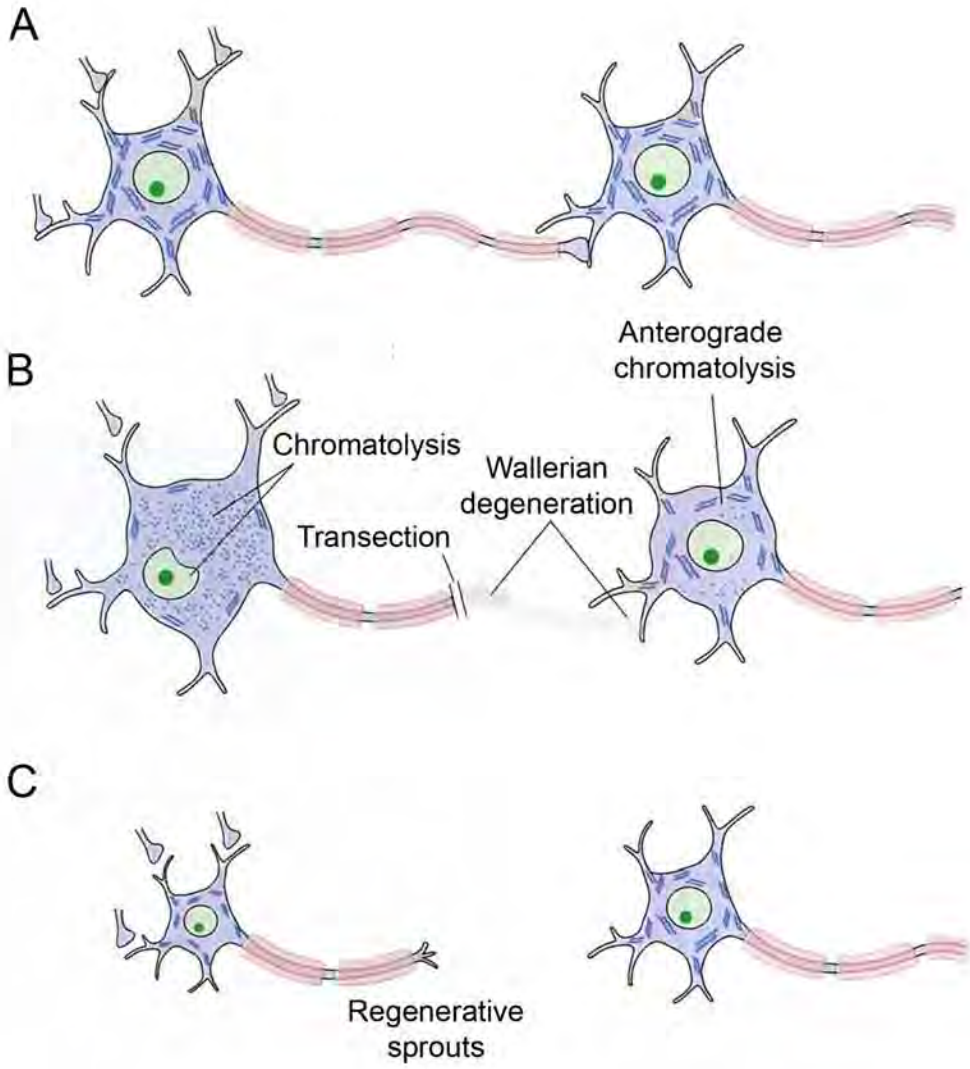


Figure 1.7 Axonal injury. Due to their relatively long-distance projection and fragility, axons are particularly vulnerable to TBI (A). Axonal injury, either as a result of primary injury or a secondary effect, results in the formation of two axonal segments Changes in the soma (chromatolysis) may culminate in cell atrophy, death or survival. Following disconnection the disconnected ends almost immediately seal over and the proximal and distal sections begin to retract away from each other and enlarge; the distal segment becomes completely separated from the cell body of origin and ultimately degenerates by a mechanism that is known as Wallerian degeneration (B). The degeneration of the distal segment also results in a loss of synaptic input to the target cells. If the cell body survives the proximal segment of an axon demonstrates a retained ability to regrow or sprout (C) (Adapted from Notle, 2009).

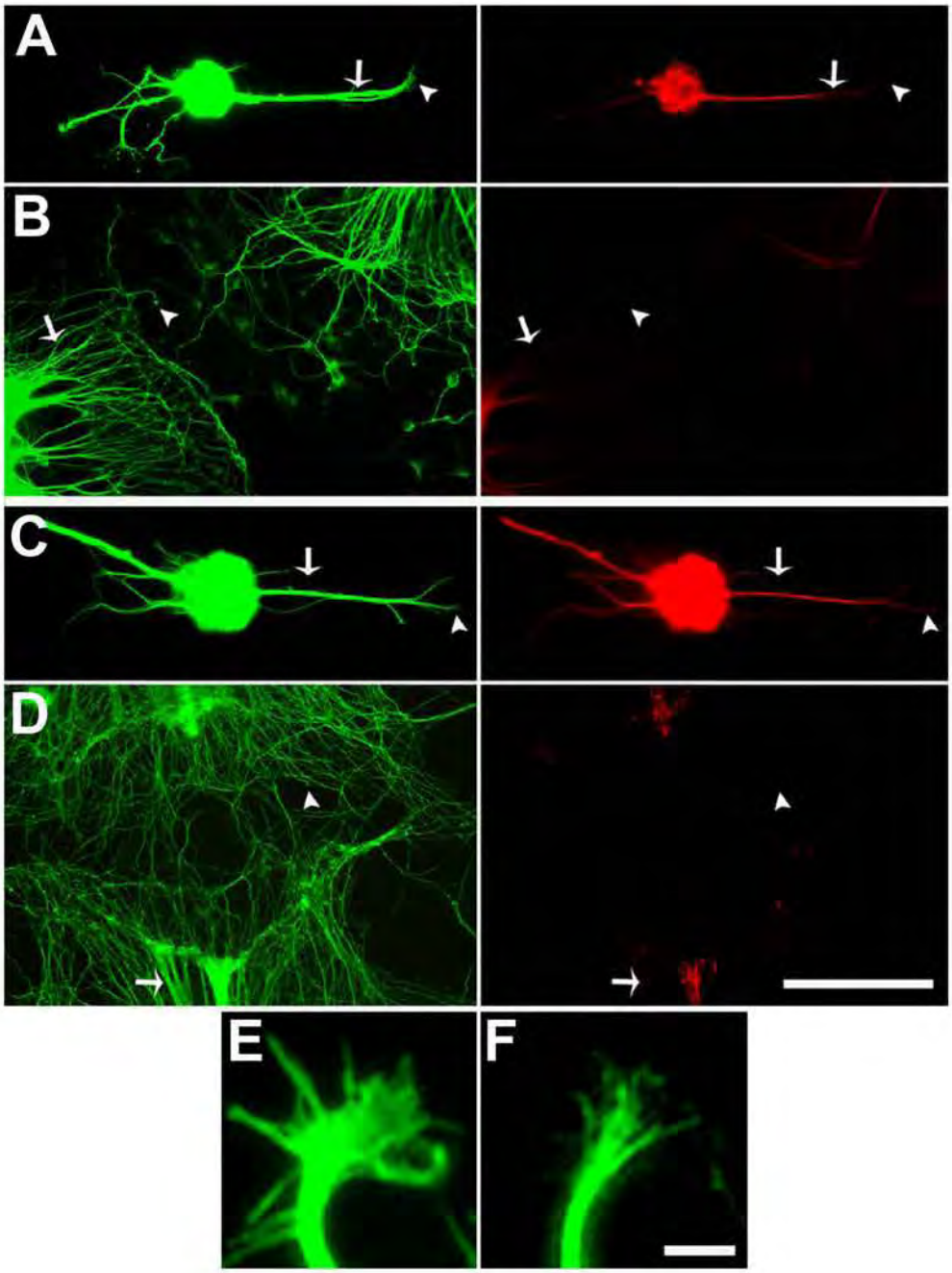

A
B
C
D
E
F

Figure 3.1 Cytoskeletal profile of cortical neurons during development and regeneration. (A and B) A neuronal aggregate at (A) 5 DIV and (B) a transected axon bundle (21 DIV) at 6 hrs PI immunolabelled for both tau (green) and MAP-2 (red). Tau was distributed throughout the axon bundles (arrow) and growth cones (arrowhead) whereas MAP-2 localisation was more restricted (arrow and arrowhead). (C and D) A typical neuronal aggregate (C) at 5 DIV and (D) a transected axonal bundle at 6hrs PI immunolabelled for both ßIII-tubulin (green) and NF-M (red). ßIII-tubulin was distributed throughout axonal bundles (arrows) and extending into growth cones (arrowheads), however NF-M was restricted to axonal shafts (arrows). (E and F) Tau labelling, co-localised with MAP-2 which was completely absent, of a growth cone (E) at 5 DIV and a (F) regenerative sprout at 6 hrs PI demonstrated that the growth cones under investigation were axonal of origin. Scale bar A-D = 10µm; E, F = 2µm

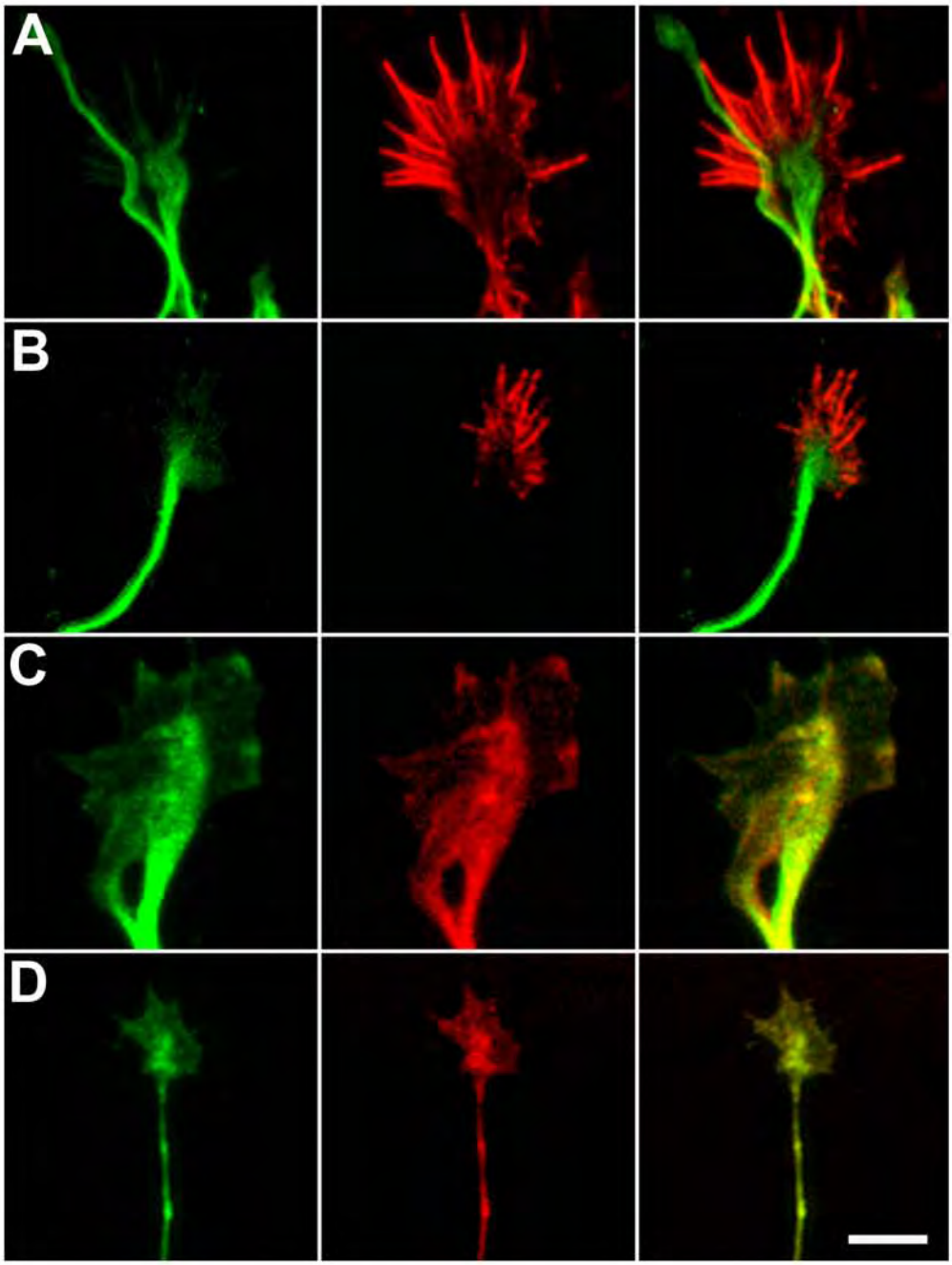

A
B
C
D

Figure 3.2 The morphology and cytoskeletal profile of growth cones of developing and regenerating axons at 5 DIV and 6 hrs PI. (A and B) Growth cones at (A) 5 DIV and (B) 6 hrs PI immunolabelled for both ßIII-tubulin (green) and ERM (red) demonstrated that ßIII-tubulin was confined predominately to the central domain of the growth cone, while total ERM was confined to the peripheral region. (C and D) Immunolabelling and staining for both tau (green) and F-actin (red) in growth cones at (C) 5 DIV and (D) 6 hrs PI indicated a widespread distribution of both cytoskeletal components throughout the growth cones. Both cytoskeletal dual-labelling combinations indicated that the 6 hrs PI growth cones were smaller, simpler structures than their developmental counterparts. Scale bar = 2µm

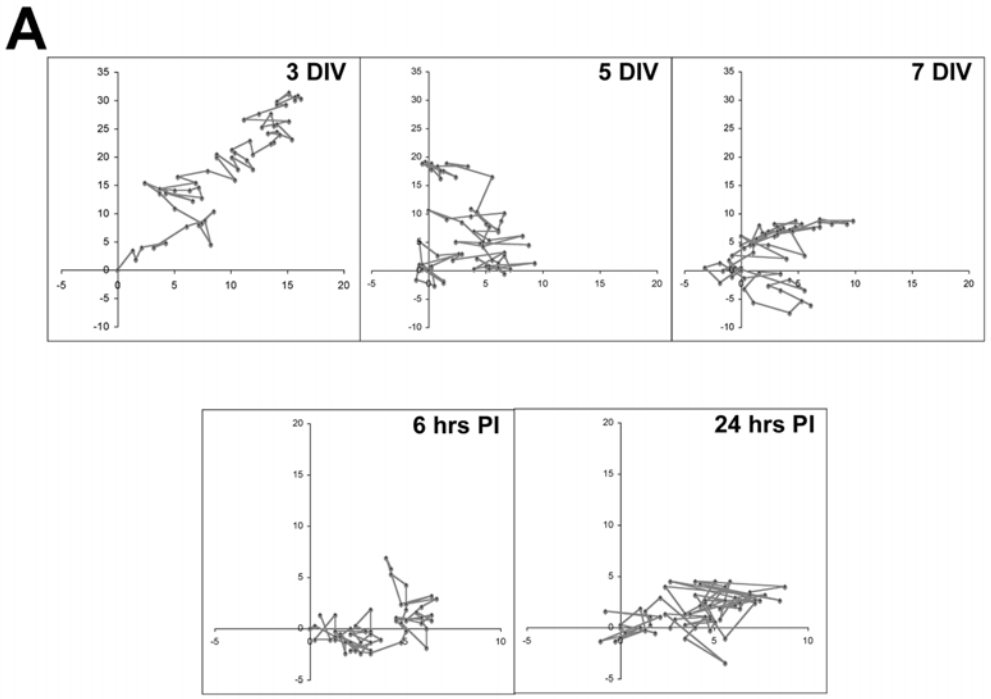

A
3 DIV
5 DIV
7 DIV
6 hrs PI
24 hrs PI
B
Axonal extension (µm)
3 DIV
5 DIV
7 DIV
6 hrs PI
24 hrs PI
C
Axonal retraction (µm)
3 DIV
5 DIV
7 DIV
6 hrs PI
24 hrs PI

Figure 3.3 Axonal motility, extension and retraction in development and regeneration. (A) X, Y graphs demonstrating growth cone motility in development (3, 5, 7 DIV) and regeneration (6, 24 hrs PI). Data points illustrate the position of the centre of the growth cone (μm), at two min intervals, over a two hrs imaging period. (B) Mean axonal extension in development and regeneration (n=25). (C) Mean axonal retraction in development and regeneration (n=25). *$p<0.05$. Error bars represent SEM.

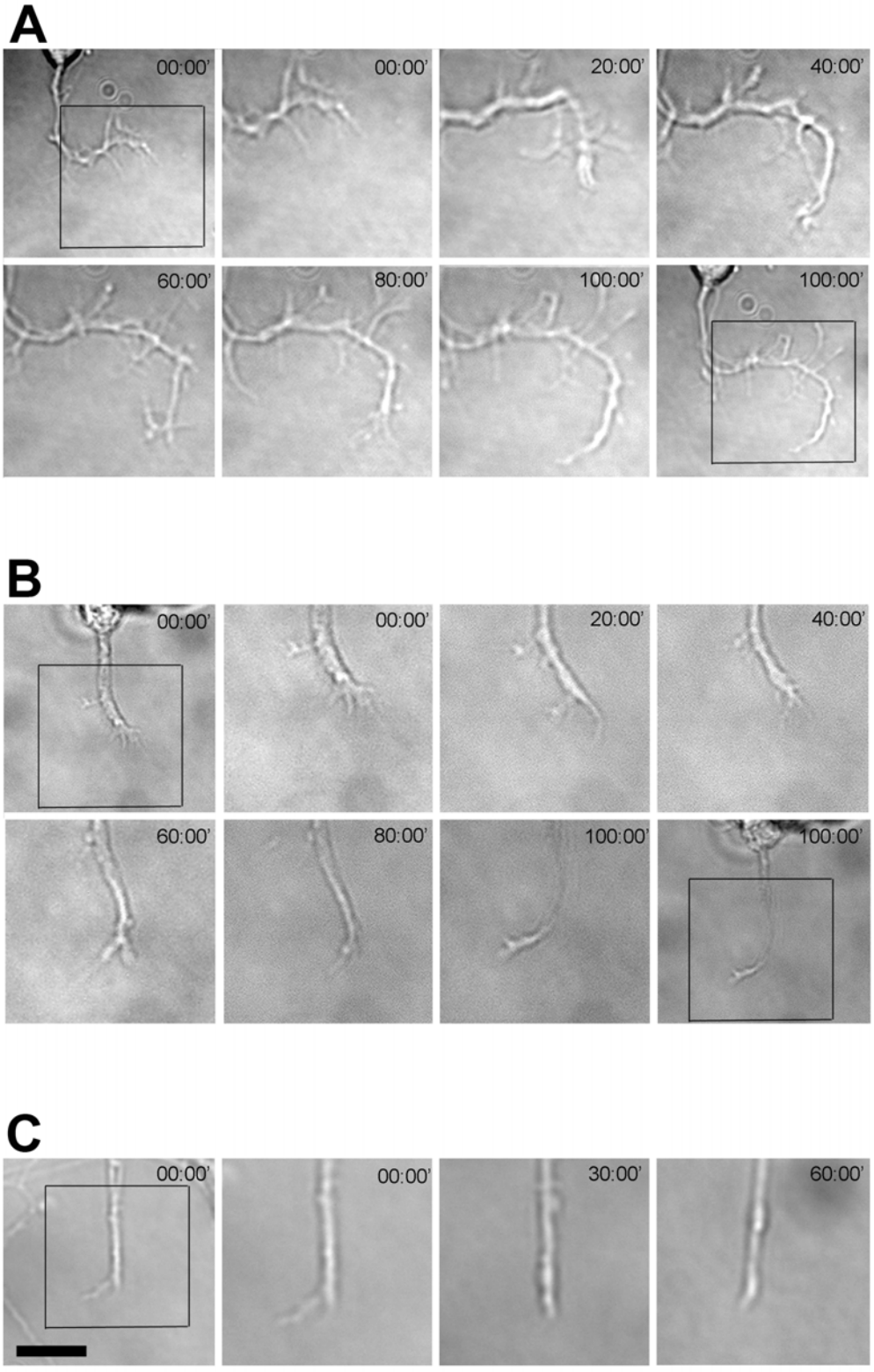

A
00:00'
00:00'
20:00'
40:00'
60:00'
80:00'
100:00'
100:00'
B
00:00'
00:00'
20:00'
40:00'
60:00'
80:00'
100:00'
100:00'
C
00:00'
00:00'
30:00'
60:00'

Figure 3.4 Growth cone dynamics in development and regeneration. (A) Live imaging of a dynamic growth cone (3 DIV) (arrowheads) over a two hrs time period indicating a range of morphological changes. (B) Live imaging of a growth cone (6 hrs PI) (arrowheads) over a two hrs time period indicating the range of morphological changes observed in development were not apparent in the regenerating growth cones. (C) Time-lapse images of an injured immature axon bundle (5 DIV) over a one hrs time period indicating no net axonal outgrowth in the immature bundles. Scale bar = 10μm, for inset 6.5μm

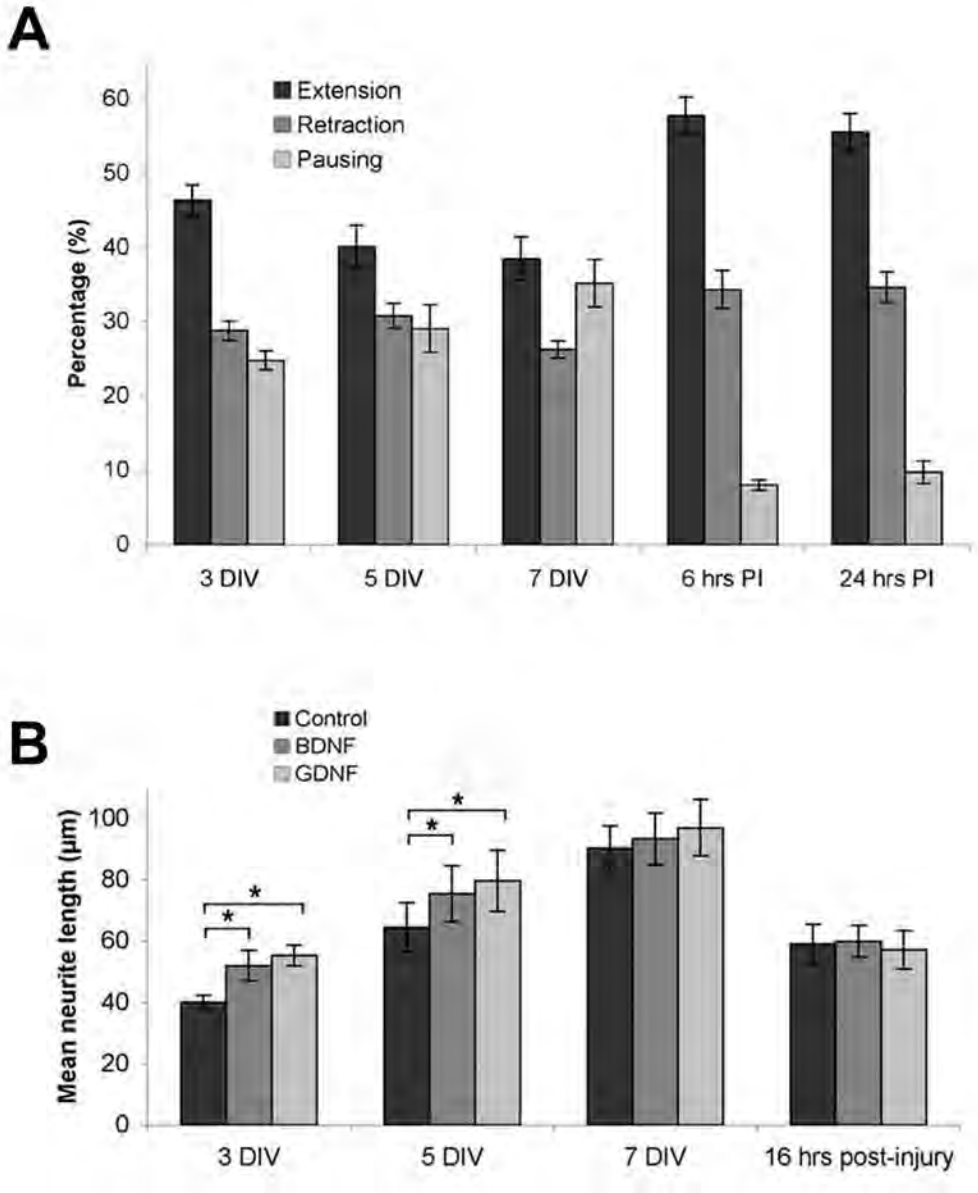

A
Extension
Retraction
Pausing
Percentage (%)
60
50
40
30
20
10
0
3 DIV
5 DIV
7 DIV
6 hrs PI
24 hrs PI
B
Control
BDNF
GDNF
Mean neurite length (µm)
100
80
60
40
20
0
*
*
*
*
3 DIV
5 DIV
7 DIV
16 hrs post-injury

Figure 3.5 Growth cone dynamics and response to growth factors. (A) The distribution of time spent extending, retracting and pausing for each imaging time point in development and regeneration (n=25). (B) The effect of BDNF and GDNF on developmental neurite outgrowth compared to post-injury regenerative sprouting. *$p<0.05$. Error bars represent SEM.

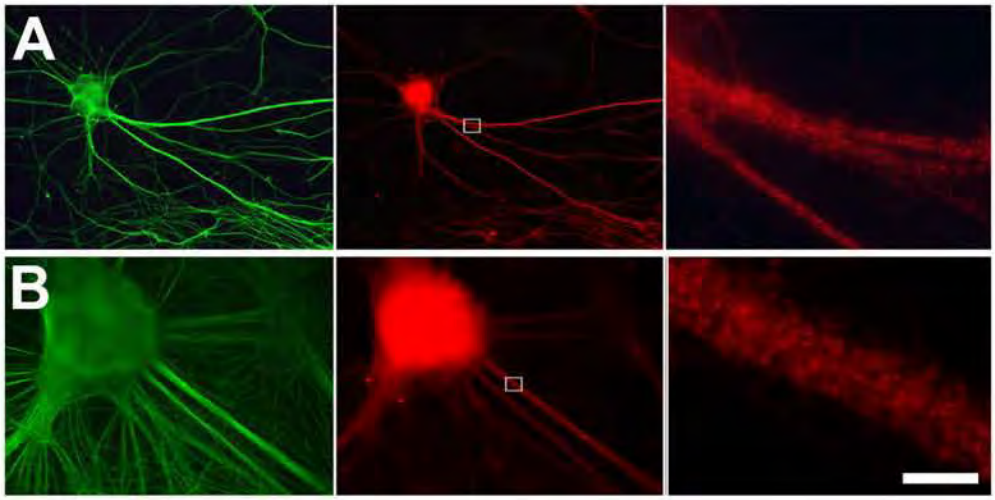

A
B

Figure 3.6 BDNF receptor localisation. (A and B) Immunolabelling with antibodies to both neuron specific βIII-tubulin (green) and the BDNF receptor TrkB (red) at (A) 5 DIV and (B) 21 DIV, indicated a widespread punctate localisation of the neurotrophin receptor throughout nerve cell bodies and processes at both ages in culture. Scale bar = 15μm, for inset 5μm

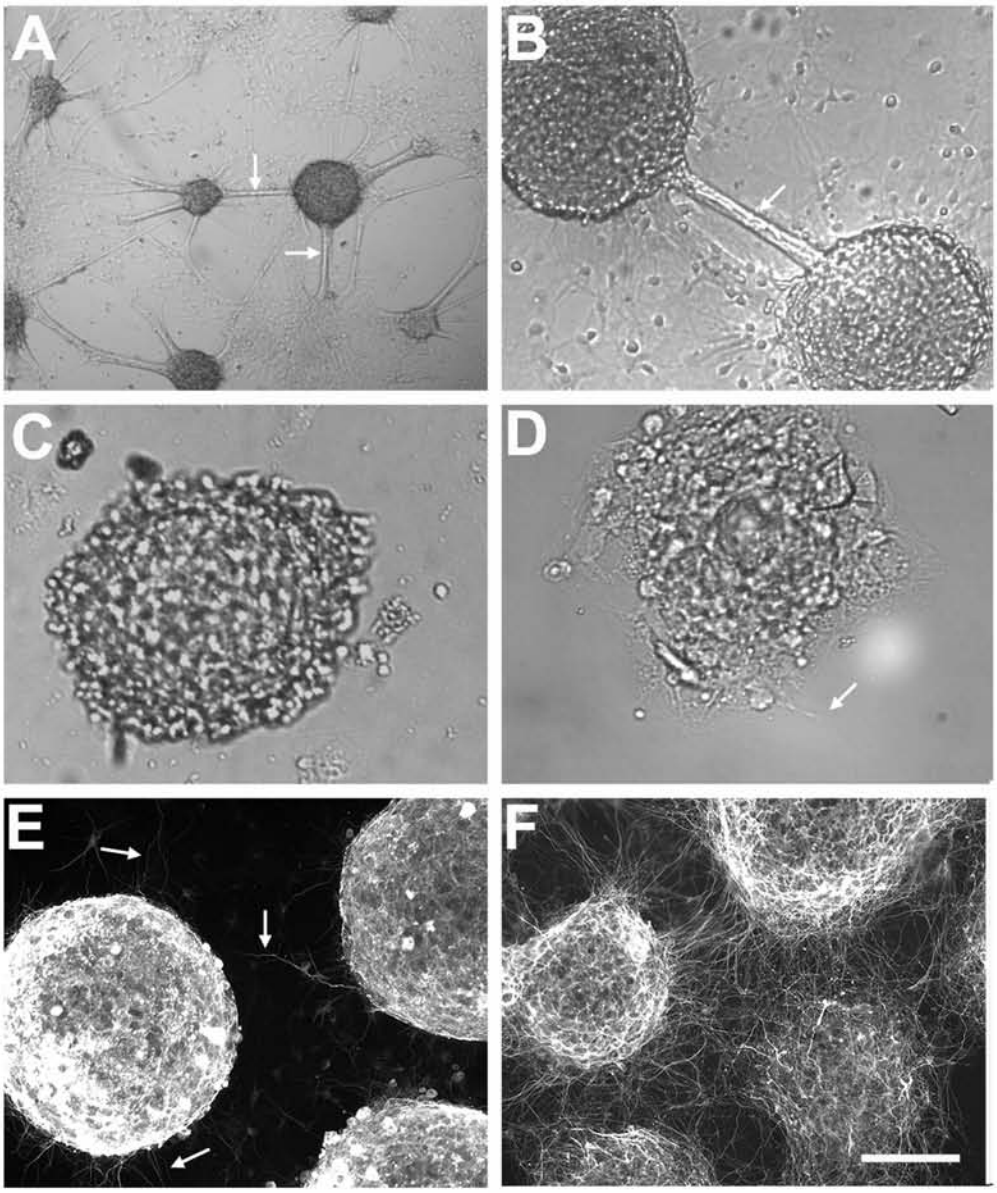

A
B
C
D
E
F

Figure 4.1 DIC and fluorescent immunolabelled images of neurons at 21 DIV. (A and B) At 21 DIV neurons formed closely associated cell clusters interconnected by axon bundles (arrows). (C) At 6 hrs post-injury neurons were still present as clusters, however, all interconnecting neurites had been stripped. (D) By 24 hrs post-injury many neurons within the cluster had extended processes, tipped with growth cone like structures (arrow). (E) Immunolabelling for the axon specific microtubule associated protein, tau, at 24 hrs post-injury, revealed numerous axonal sprouts emanating from the cell clusters (arrows). (F) By 7 DPI, single axonal (tau immunoreactive) sprouts connected with surrounding cell clusters. Scale bar: A = 100μm, B-E = 25 μm

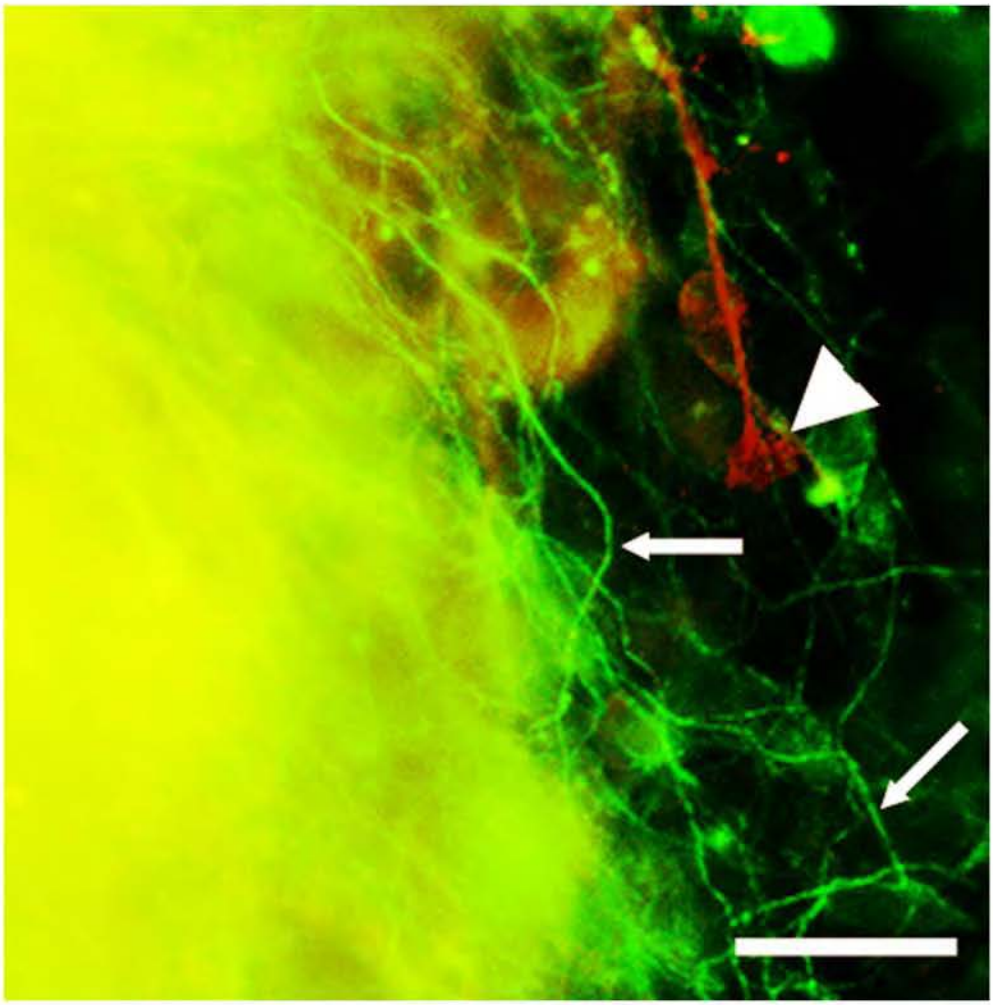


Figure 4.2 Confocal microscopy at 24 hrs post-injury. A subset of the sprouts within the cell cluster were immunopositive for calretinin (red, arrowhead), with a further larger group of sprouts extending out form the cell cluster that were immunoreactive for SM312 (green, arrows). No colocalisation between these markers was evident. Scale bar =15μm

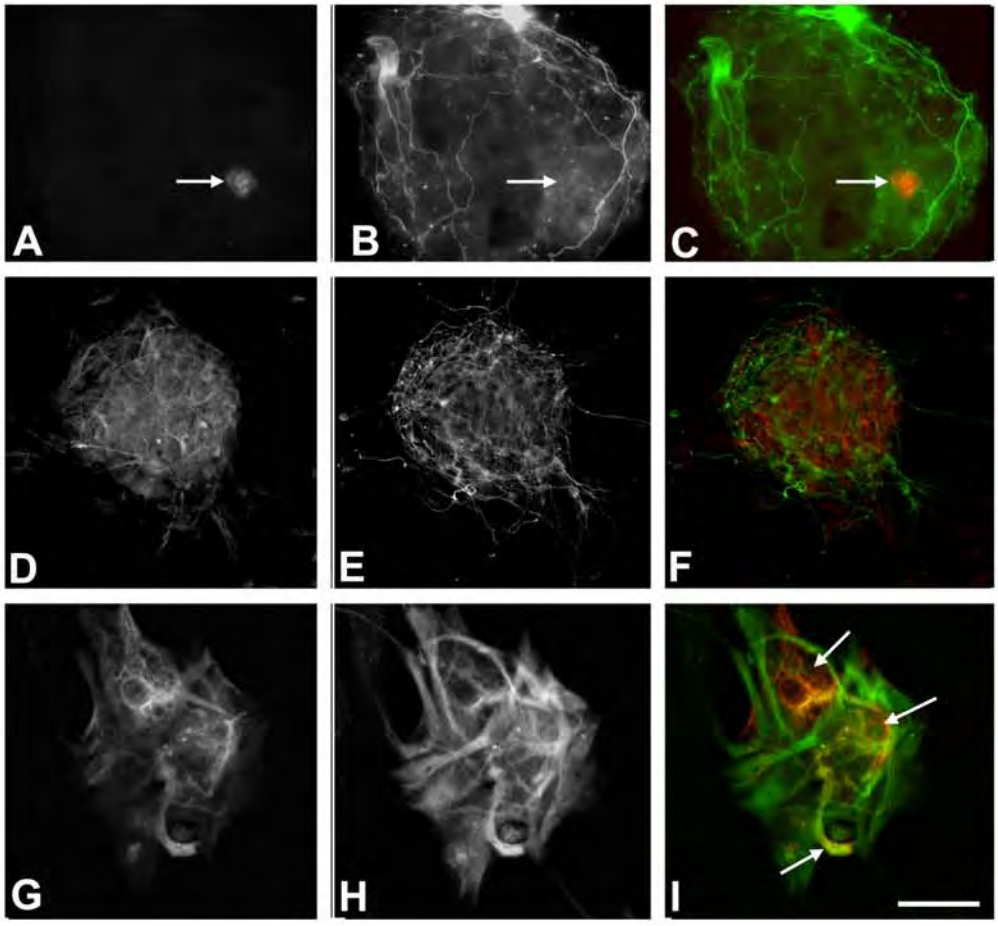

A
B
C
D
E
F
G
H
I

Figure 4.3 Confocal microscopy of immunocytochemical labelling for cell-type-specific proteins and markers of neurogenesis in relatively mature neurons after structural injury. (A-C) (A) Labelling for the thymidine analogue, BrdU, (B) in combination with a neuronal marker tau, (C) indicated that the structurally injured neurons under investigation did not incorporate BrdU (arrow). (D-F) (D) Labelling with the neural precursor marker nestin, (E) in conjunction with tau, indicated the presence of precursors cells (nestin immunopositive) present after injury, (F) however the labelling did not co-localise with tau. (G-I) Colocalisation between (G) nestin and (H) GFAP immunolabelling, (arrows, (I)) indicated that the precursor cells present within the cell clusters after injury, were of glial origin. Scale bar: A-C= 15μm, D-I = 20μm

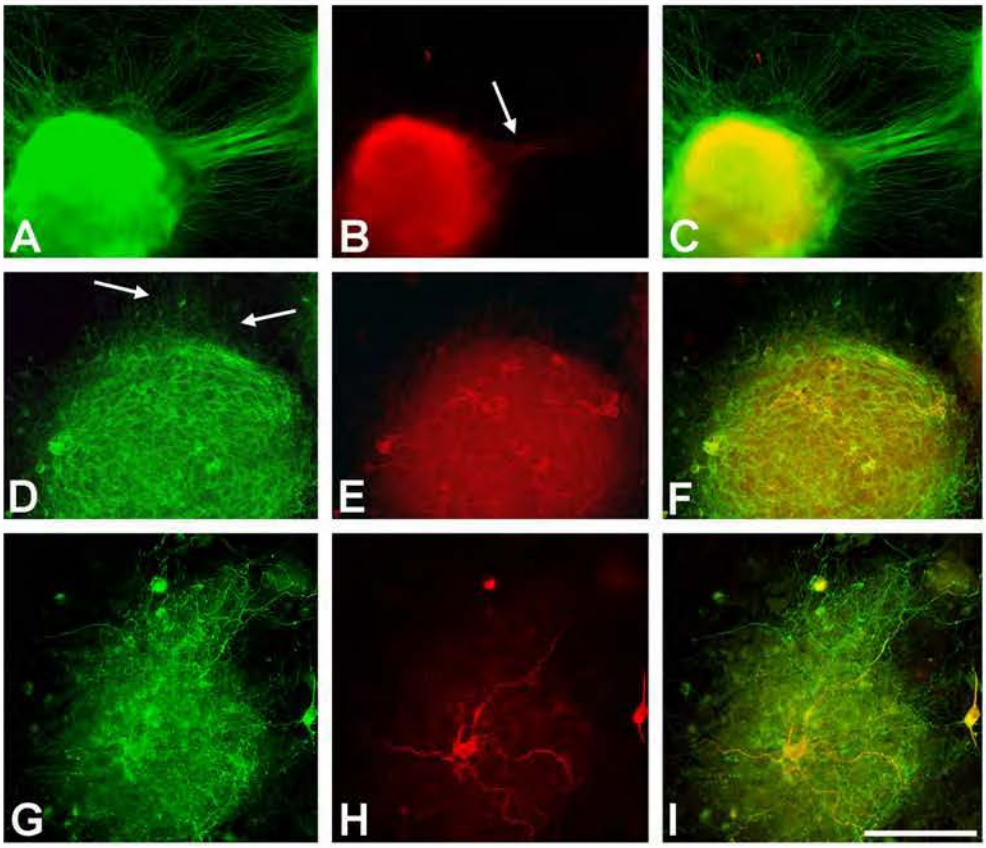

A
B
C
D
E
F
G
H
I

Figure 4.4 Tau and MAP-2 expression in cortical neurons at 21 DIV and post-injury. (A-C) At 21 DIV (A) tau was distributed throughout cell clusters and neuronal processes, (B) whereas MAP-2 was confined to cell clusters and processes extending for only a short distance (arrows). (D-F) Similarly, at 24hrs post-injury tau was distributed throughout cell clusters and (D) newly extending neurites (arrows), (E) whereas MAP-2 remained in the cell clusters. (F) Confocal microscopy revealed no colocalisation between the two markers in the sprouting neurites. (G-I) High power confocal microscopy revealed (G) distinct distal tau immunolabelled axons and (H and I) single neurons within the clusters with multiple dendrites expressing only MAP-2. Scale bar: A-C = 50 μm, D-I = 25 μm

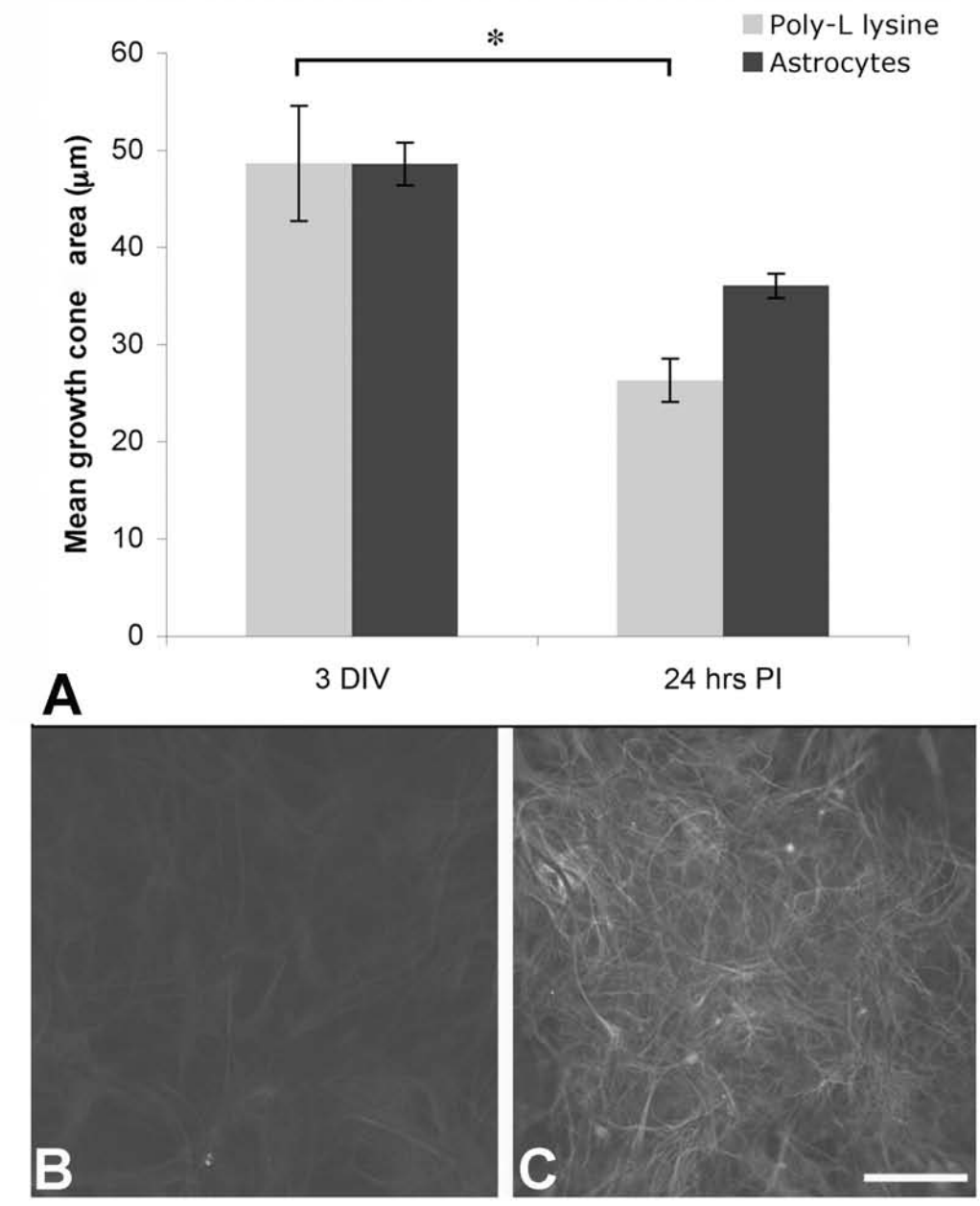

Poly-L lysine
Astrocytes
*
Mean growth cone area (µm)
60
50
40
30
20
10
0
3 DIV
24 hrs PI
A
B
C

Figure 4.5 Growth cone area for developing axons at 3 DIV in comparison to regenerative axons at 24 hrs post-injury. (A) The mean growth cone area was significantly (*$p<0.05$) greater in developing neurites at 3 DIV compared to regenerative neurites at 24 hrs post-injury. (A) There were no significant differences for mean growth cone area on astrocytes in comparison to poly-L-lysine for developing and regenerating growth cones. (B and C) Regenerating neurons (24 hrs post-injury), (C) when plated on to a monolayer of astrocytes, induced an increase in the fluorescence intensity of GFAP labelling and an alteration in astrocyte morphology, (B) in comparison to an astrocyte monolayer without injured neurons or developing neurons. Scale bar = 20μm

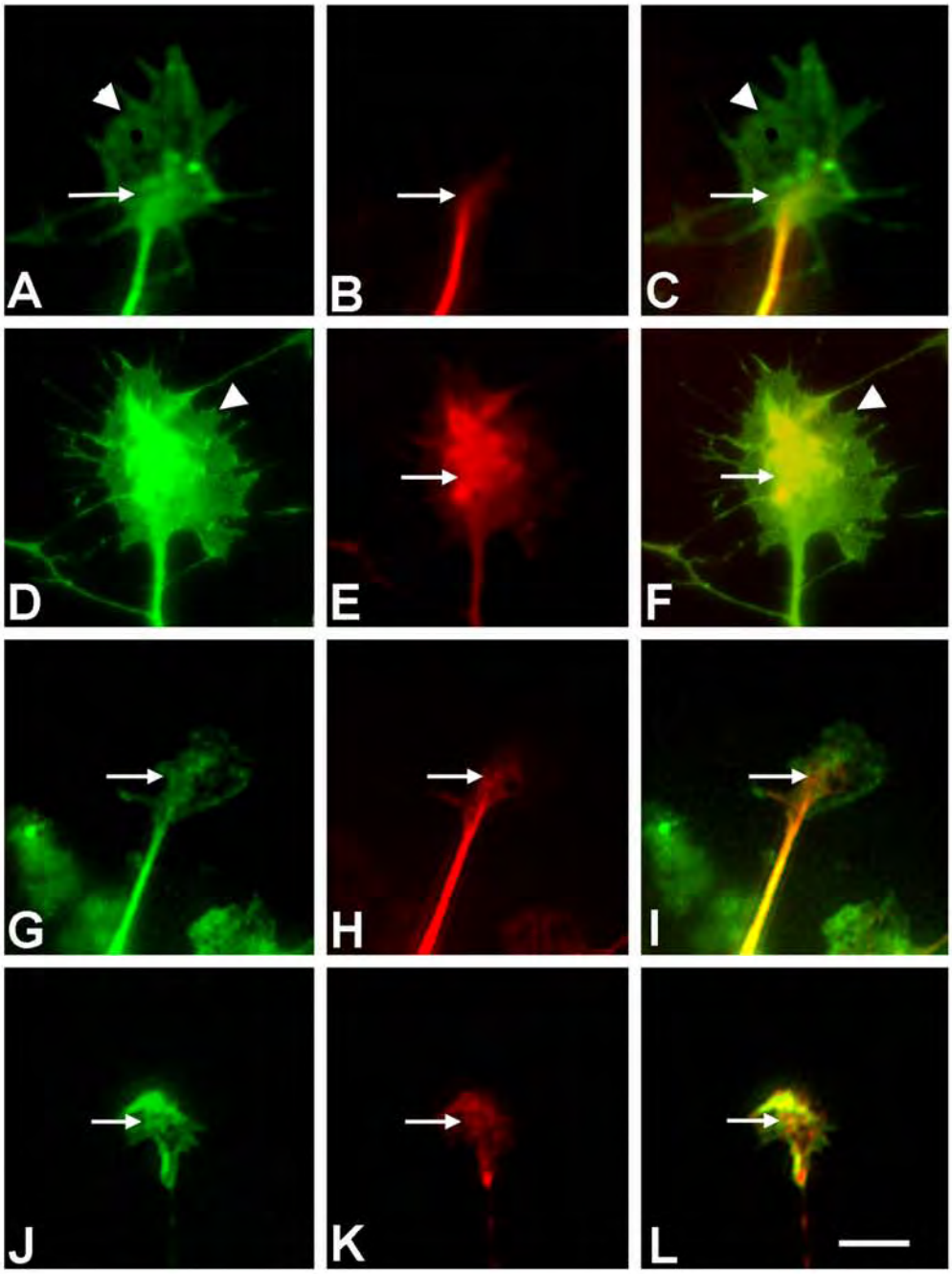

A
B
C
D
E
F
G
H
I
J
K
L

Figure 4.6 Immunocytochemical profile of growth cones of developing and regenerating axons at 3 DIV and 24 hrs post-injury. (A-F) Growth cones at 3 DIV were large and showed extensive labelling (A and C, green) for tau and (D and F, green) staining for F-actin, using phalloidin that was concentrated in the central domain (arrow) of the growth cone but crossed into the peripheral domain (arrowhead). At 24 hrs post-injury the growth cones were smaller and (G,I, green) the tau and (J,L, green) F-actin expression was concentrated in the central domain (arrow), with no peripheral domain labelling evident. (B,C and H,I, red) Alpha-acetylated tubulin was confined predominately to the central domain of the growth cone and neurite shaft in both (B,C) developmental and (H,I) regenerative growth cones. (E,F and K,L, red) Similarly, GAP-43 expression was concentrated in the growth cones central domains in both (E,F) development at 3 DIV and (K,L) 24 hrs post-injury (arrows). Scale bar = 5 μm

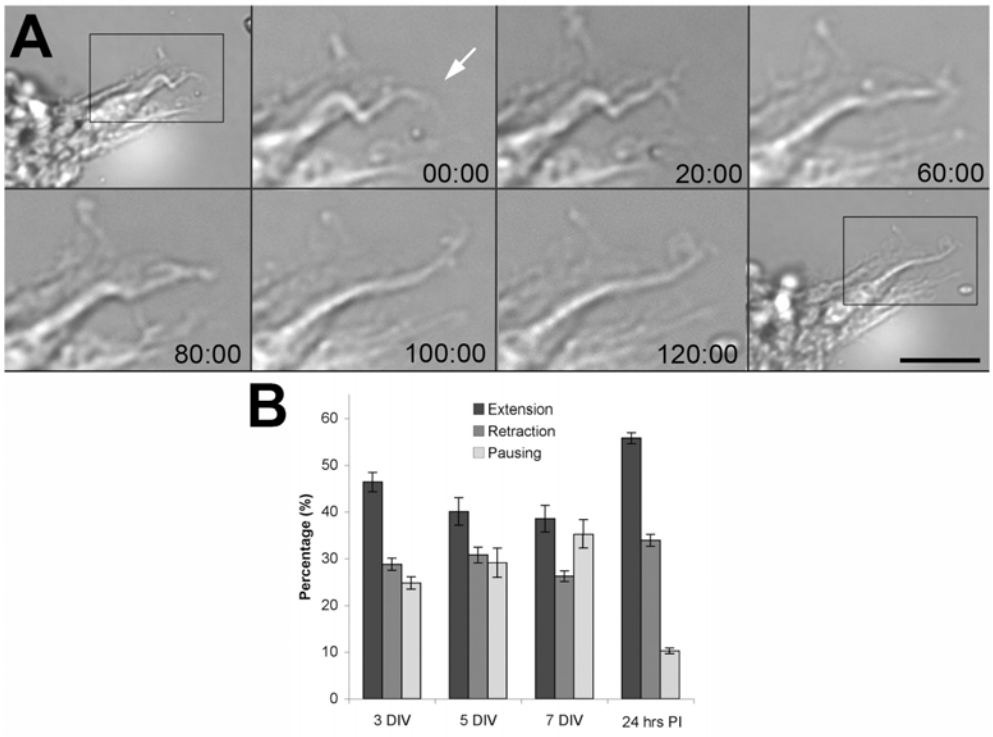

A
00:00
20:00
60:00
80:00
100:00
120:00
B
Extension
Retraction
Pausing
Percentage (%)
60
50
40
30
20
10
0
3 DIV
5 DIV
7 DIV
24 hrs PI

Figure 4.7 Growth cone dynamics in development and attempted regeneration. (A) An example of live imaging of a dynamic growth cone (arrow) (24 hrs PI) at 20 min intervals for two hrs. (B) The distribution of time growth cones spent extending, retracting and pausing (assessed every min for 120 mins) in development and at 24 hrs post-injury (n=30). Growth cones on the tips of regenerating axons spent significantly ($p<0.05$) more time extending and less time pausing then developing growth cones at 3, 5 and 7 DIV. Scale bar = 10μm, for inset 6.5 μm

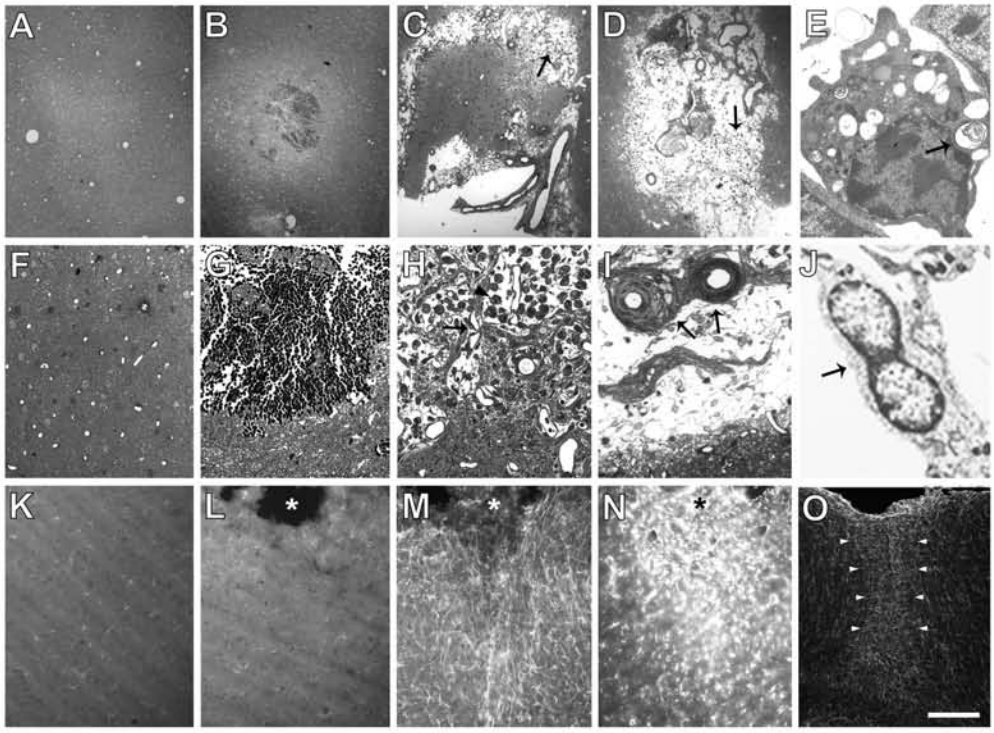

A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 5.1 Immunofluorescent, light and transmission electron microscopy following focal brain injury. (A and F) Resin sections cut in the horizontal plain from the control non-injured cortex showed that the neuropil of the somatosensory cortex of the right hemisphere was dense unbroken structure comprising of cell bodies and their processes and blood vessels. (B and G) At 1 DPI a discreet, circular injury site was clearly visible in the horizontal sections. (G) The injury site was filled with red blood cells indicating there had been bleeding within the injury site. (C) By 7 DPI the injury site (arrow) had dramatically increased in size. (H) The red blood cells had cleared and the injury site was populated with cells (arrowheads) and processes, the area innervated with new blood vessels (arrows). (D) The injury site at 14 DPI remained large in comparison to that at 1 DPI and had become more densely infiltrated with cells and their processes, in comparison to 7 DPI. (I) At 14 DPI blood vessels permeated the injury tract (arrows). (E) TEM analysis at 7 and 14 DPI demonstrated that the injury site was infiltrated with activated microglia with prominent inclusion bodies (arrow). (J) Proliferating cells (arrow denotes a dividing nuclei) were also present within the lesion site at 7 DPI. (K) Immunofluorescent labelling of horizontal sections with an antibody to GFAP demonstrated minimal expression in the uninjured brain. (L) There was little GFAP immunoreactivity around the injury site at 1 DPI. (M) At 7 DPI there was a localised increased expression of GFAP around the injury site. (N) By 14 DPI there was substantial GFAP reactivity around the injury site that extended to 500μm away from the injury. (O) Coronal sections labeled with GFAP demonstrated that by 14 DPI a dense glial scar had formed within the injury tract. Scale bar: A-D = 250μm, F-I = 100μm, E and J = 2μm, K- N = 150μm, O = 500μm, asterisk in L-N denotes injury site

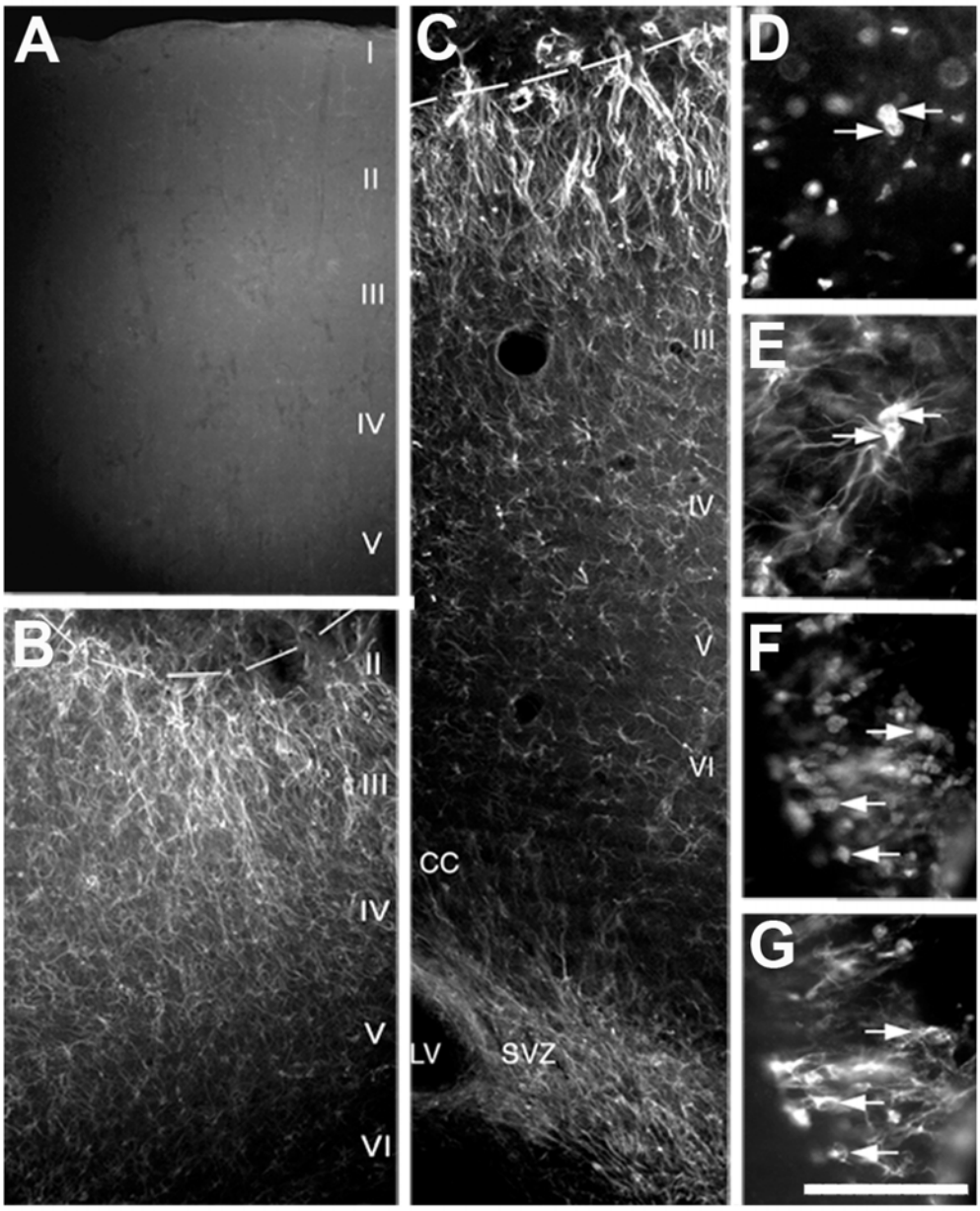

A
I
II
III
IV
V
B
II
III
IV
V
VI
C
II
III
IV
V
VI
CC
LV
SVZ
D
E
F
G

Figure 5.2 Immunofluorescence labelling for nestin within the cortex at 7 DPI. Nestin immunoreactive cells were not evident in the grey matter of the neocortex of control non-injured brains (A), but by 7 DPI, were abundant within the lesioned tissue (B). Nestin immunoreactive profiles were present in tissue extending from the SVZ and corpus callosum up to the injury site (C). Double immunofluorescence labelling for BrdU, administered at 1 DPI, (D) and nestin (E) demonstrated localisation of these markers to the same cells within peri lesion tissue. Double labelling for PCNA (F) relative to nestin (G) confirmed the proliferation of nestin-labelled cells within the SVZ. Scale bar: A and B = 100μm, C = 800μm, D-G = 50μm

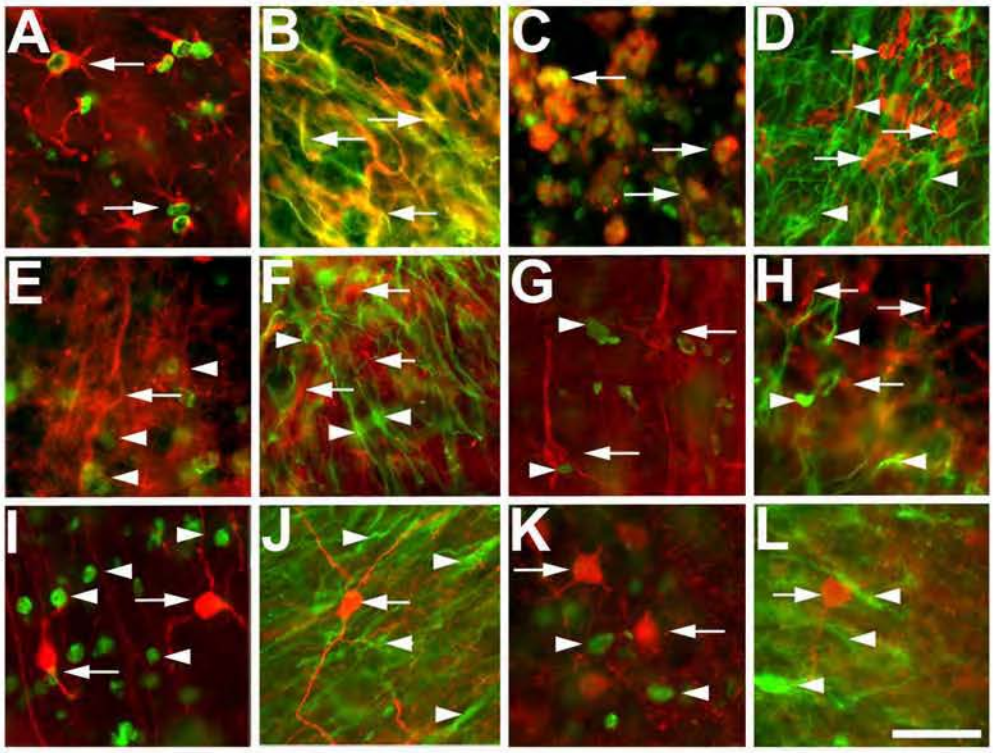

A
B
C
D
E
F
G
H
I
J
K
L

Figure 5.3 Double immunofluorescence labelling for BrdU and nestin (green) relative to astrocyte, microglial/macrophage and neuronal markers (red) in cortical peri lesion tissue at 7 DPI. Numerous GFAP labelled cells also contained BrdU immunoreactive nuclei (A), indicating that a proportion of the reactive astrocyte population proliferates in response to injury. Nestin labelling exclusively co-localised with GFAP (B) within the peri lesion tissue. Ferritin immunopositive microglia/macrophages were also mitotically active following injury, with a number of cells double labelling with BrdU (C). Nestin labelling was not present within the ferritin positive microglia/macrophages (D). Colocalisation with BrdU or nestin was not observed for any of the neuronal markers including NF-M, (E and F, double labeled for BrdU and nestin, respectively), α-internexin (G and H, double labeled for BrdU and nestin, respectively), calretinin (I and J, double labeled for BrdU and nestin, respectively) and parvalbumin (K and L, double labeled for BrdU and nestin respectively). Scale bar = 50μm

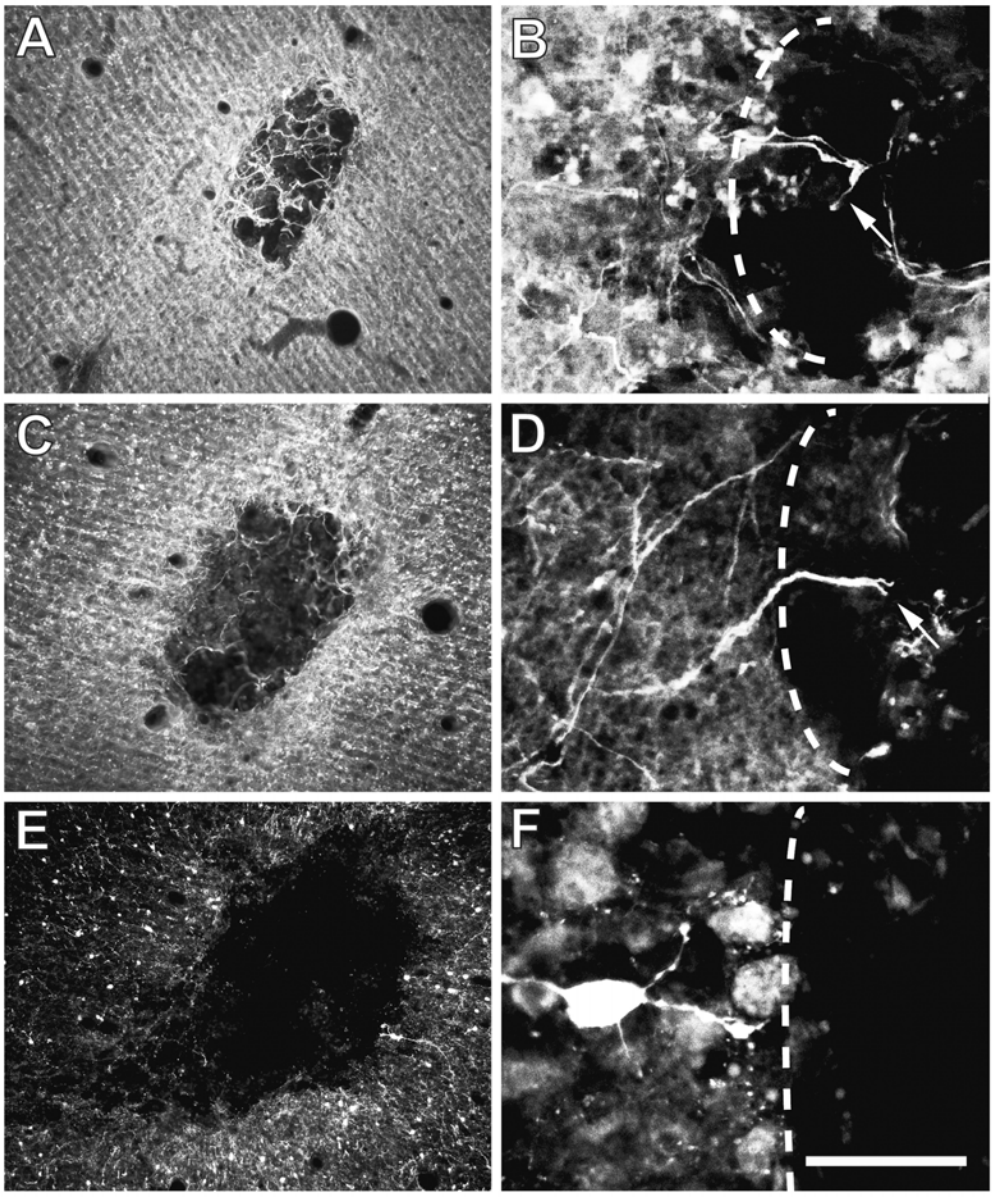

A
B
C
D
E
F

Figure 5.4 Immunofluorescent microscopy and confocal microscopy on horizontal sections at 7 DPI. (A, C) Immunolabelling with the pyramidal neuron markers (A) SMI312 and (C) α-internexin showed an increased immunoreactivity around the injury site. (B, D) High power confocal images demonstrated that fine sprouts (arrows), (immunopositive for (B) SMI312 and (D) α-internexin, were transversing the injury site. However, neurons immunopositive for the interneuronal marker calretinin did not demonstrate the same response. (E and F) Immunolabelling for calretinin was confined to the intact tissue. (F) Furthermore high power confocal images confirmed no sprouting response present beyond the lesion edge. Scale bar: A, C, E = 200μm, B, D, F = 50μm, dotted line denotes edge injury site

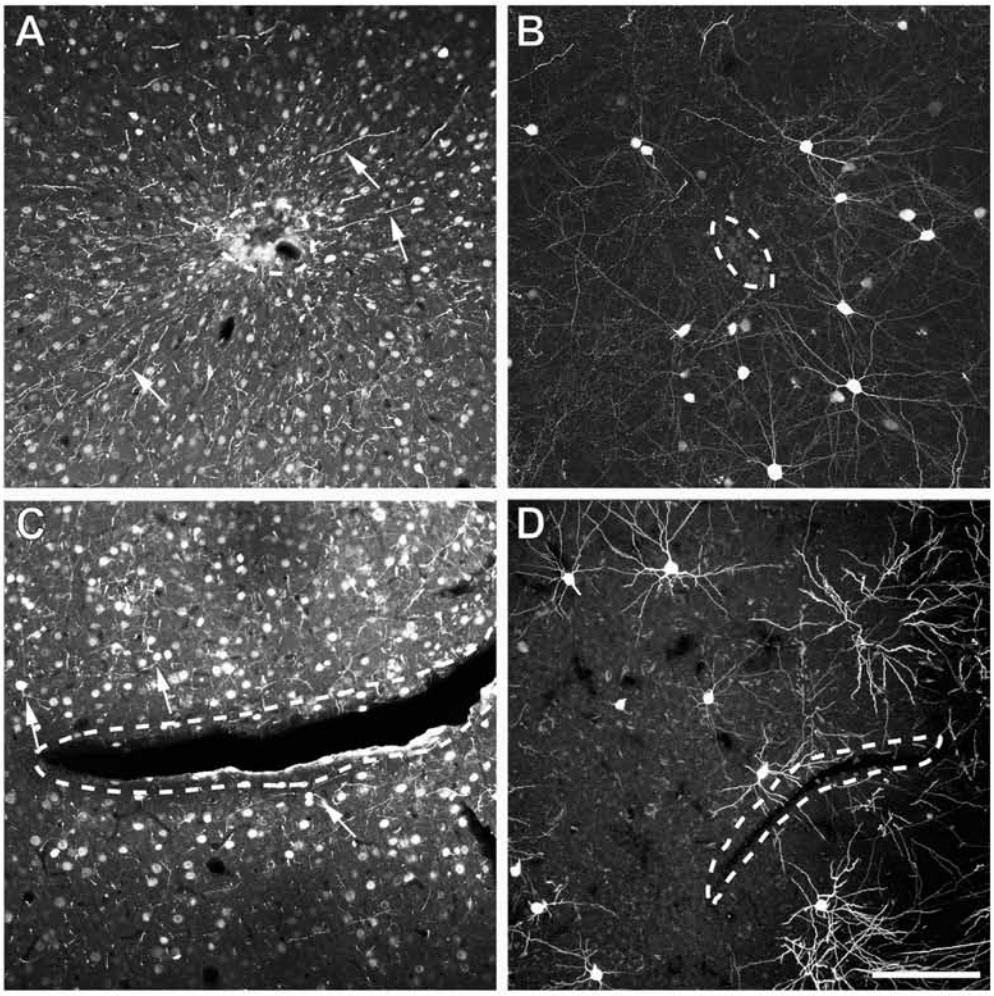

A
B
C
D

Figure 5.5 Confocal images for calretinin immunolabelling and GFP localisation at 14 DPI. (A and B) Confocal images for (A) calretinin and (B) GFP horizontal sections at 14 DPI demonstrated that the injury site had filled, forming a dense tissue core. (C) Horizontally projecting dendrites (arrows) of calretinin labelled interneurons can be clearly visualised around the injury site in sham inured control sections. (A) At 14 DPI the horizontally projecting dendrites of calretinin labeled interneurons had realigned around the injury site (arrows), as demonstrated with confocal microscopy. (B, D) Comparison between GFP localisation at (B) 14 DPI and (D) control sham injured section revealed no detectable differences. Scale bar = 150μm, dotted line denotes edge of injury site

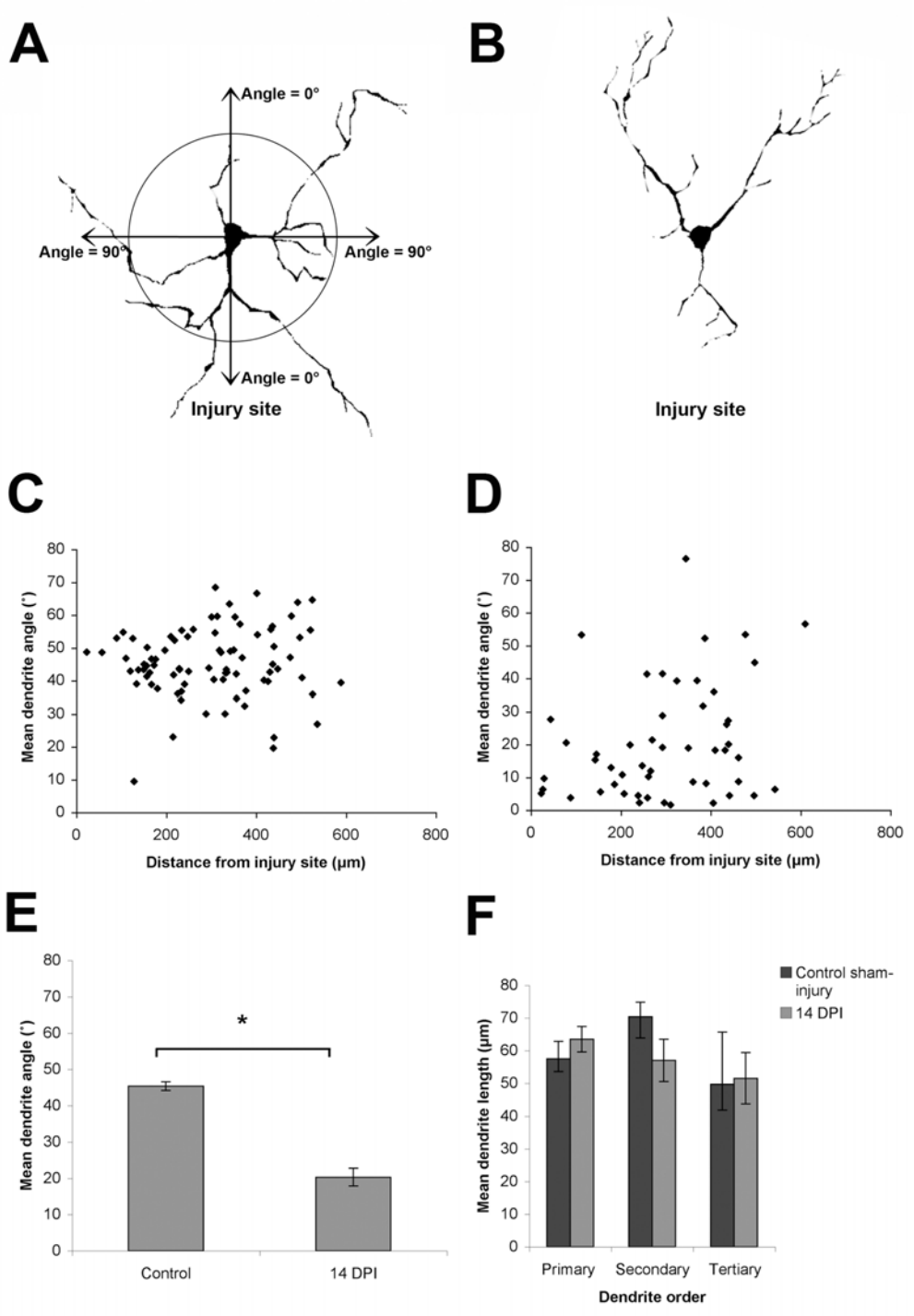
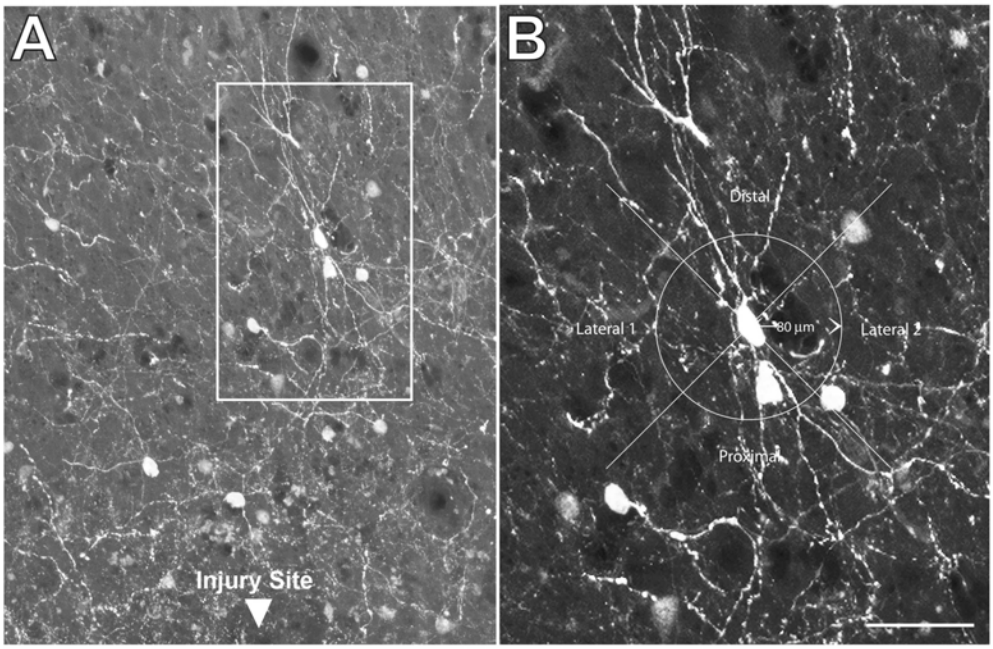

A
Angle = 0°
Angle = 90°
Angle = 90°
Angle = 0°
Injury site
B
Injury site
C
Mean dendrite angle (°)
Distance from injury site (µm)
D
Mean dendrite angle (°)
Distance from injury site (µm)
E
Mean dendrite angle (°)
*
Control
14 DPI
F
Mean dendrite length (µm)
Control sham-injury
14 DPI
Primary
Secondary
Tertiary
Dendrite order

Figure 5.6 Quantitation of horizontally projecting neurites of calretinin-immunolabelled cells at 14 DPI. Horizontally projecting dendrites were visualised using flattened z-stacks. (A and B), illustrate representative flattened z-stacks for calretinin immunolabelled neurons in (A) control sham-injured and (B) injured horizontal sections. (C and D) Plot distribution for (C) the control sham-injured and (D) injured populations in relation to the distance of the cell from the injury site further demonstrated (D) the distinct shift in dendrite angle bias of the injured neurons, with a trend for this bias to be increased away from the injury site, however linear regression demonstrated that this trend was not significant ($p=0.05$). (E) The mean of dendrite angle bias for each cell was significantly ($p<0.01$) decreased at 14 DPI in comparison to control. (F) The quantitation of the mean neurite length of primary secondary and tertiary branches at 14 DPI and in sham injured control revealed no significant differences between 14 DPI and control. Error bars denote SEM.


A
Injury Site
B
Distal
Lateral 1
80 µm
Lateral 2
Proximal

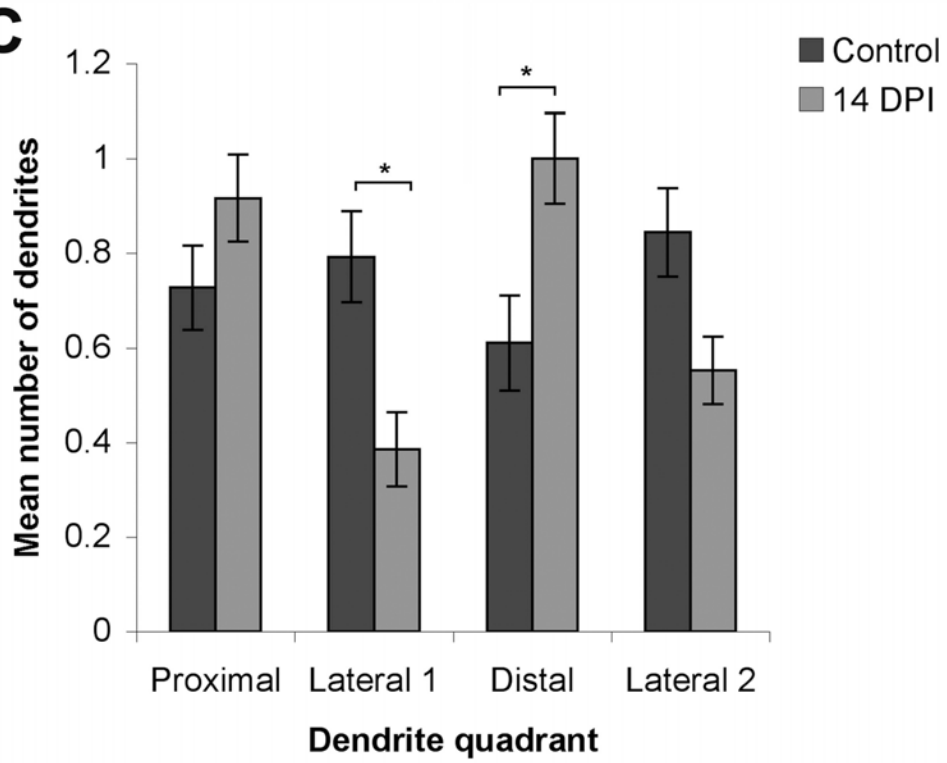

C
Control
14 DPI
Mean number of dendrites
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Proximal
Lateral 1
Distal
Lateral 2
Dendrite quadrant

Figure 5.7 Polarity quantitation of horizontally projecting neurites of calretinin-immunolabelled cells, at 14 DPI. (A and B inset) A template comprised of four quadrants was layered over each neuron under investigation and the quadrant in which each dendrite intersected the 80μm line (B, arrow) recorded. (C) The quantitation of dendrite orientation at 14 days post injury demonstrated a significant ($p<0.01$) difference in number of dendrites in the distal quadrant away from the injury site, in comparison to dendrites on neurons in control sham-injured tissue. Error bars denote SEM. Scale bar = 200μm, 100μm for inset

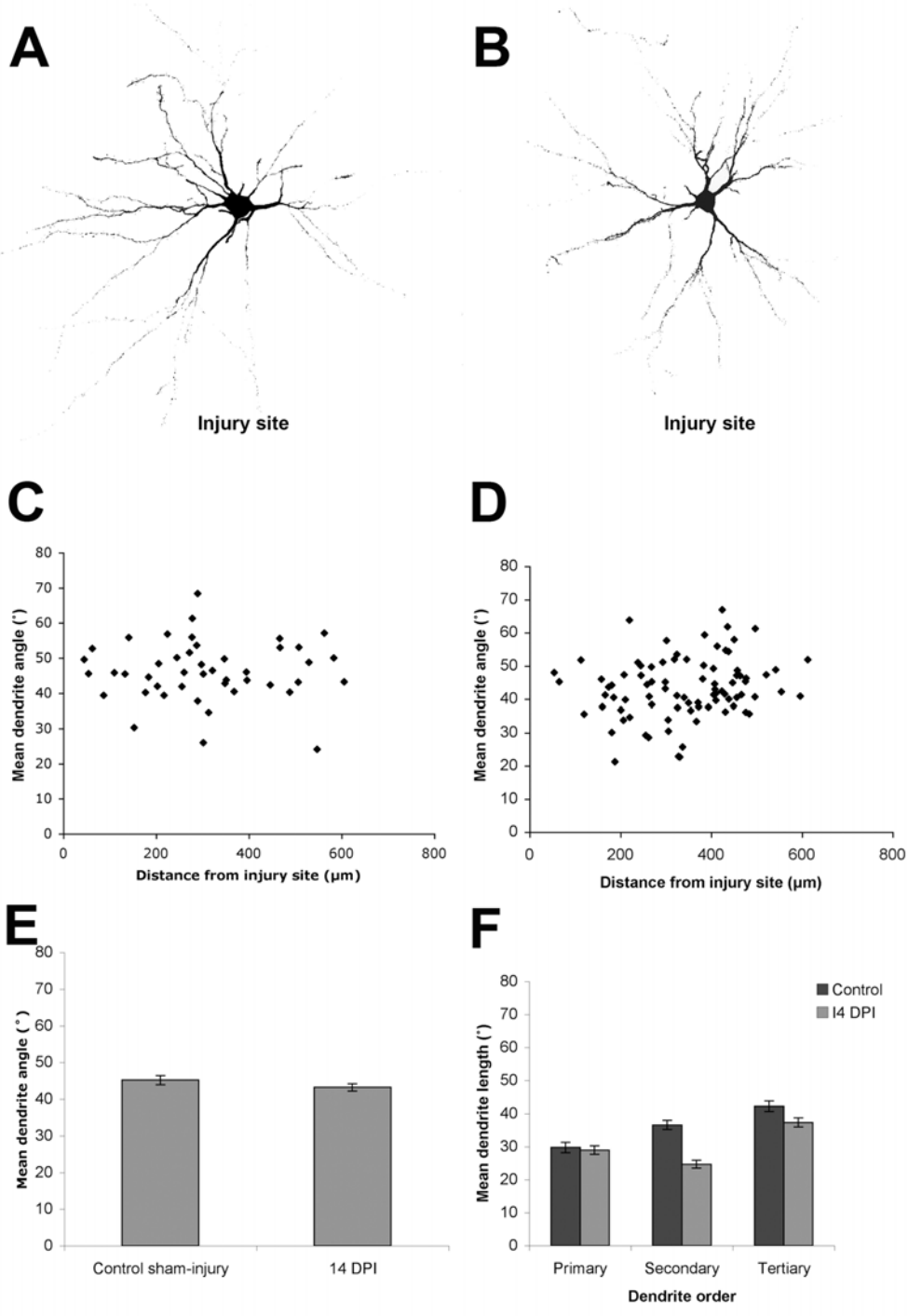

A
B
Injury site
Injury site
C
D
Mean dendrite angle (°)
Distance from injury site (µm)
Mean dendrite angle (°)
Distance from injury site (µm)
E
F
Mean dendrite angle (°)
Control sham-injury
14 DPI
Mean dendrite length (°)
Control
I4 DPI
Primary
Secondary
Tertiary
Dendrite order

Figure 5.8 Horizontally projecting neurites of GFP expressing pyramidal cells underwent no significant change in dendrite orientation or mean length, at 14 DPI. Horizontally projecting dendrites were visualized using flattened z-stacks. A and B, illustrate representative flattened z-stacks for GFP expressing neurons in (A) control sham-injured and (B) injured horizontal sections. (C and D) Plot distribution for the (C) control populations and (D) injured populations in relation to the distance from the injury site further demonstrated no distinct shift in dendrite angle bias of the injured neurons. (E) There was no significant difference in the mean dendrite angle bias between 14 DPI and sham injured control for this subpopulation of neurons. (F) The quantitation of the mean neurite length of primary secondary and tertiary branches at 14 DPI and sham injured control revealed no significant differences between 14 DPI and control. Error bars denote SEM.

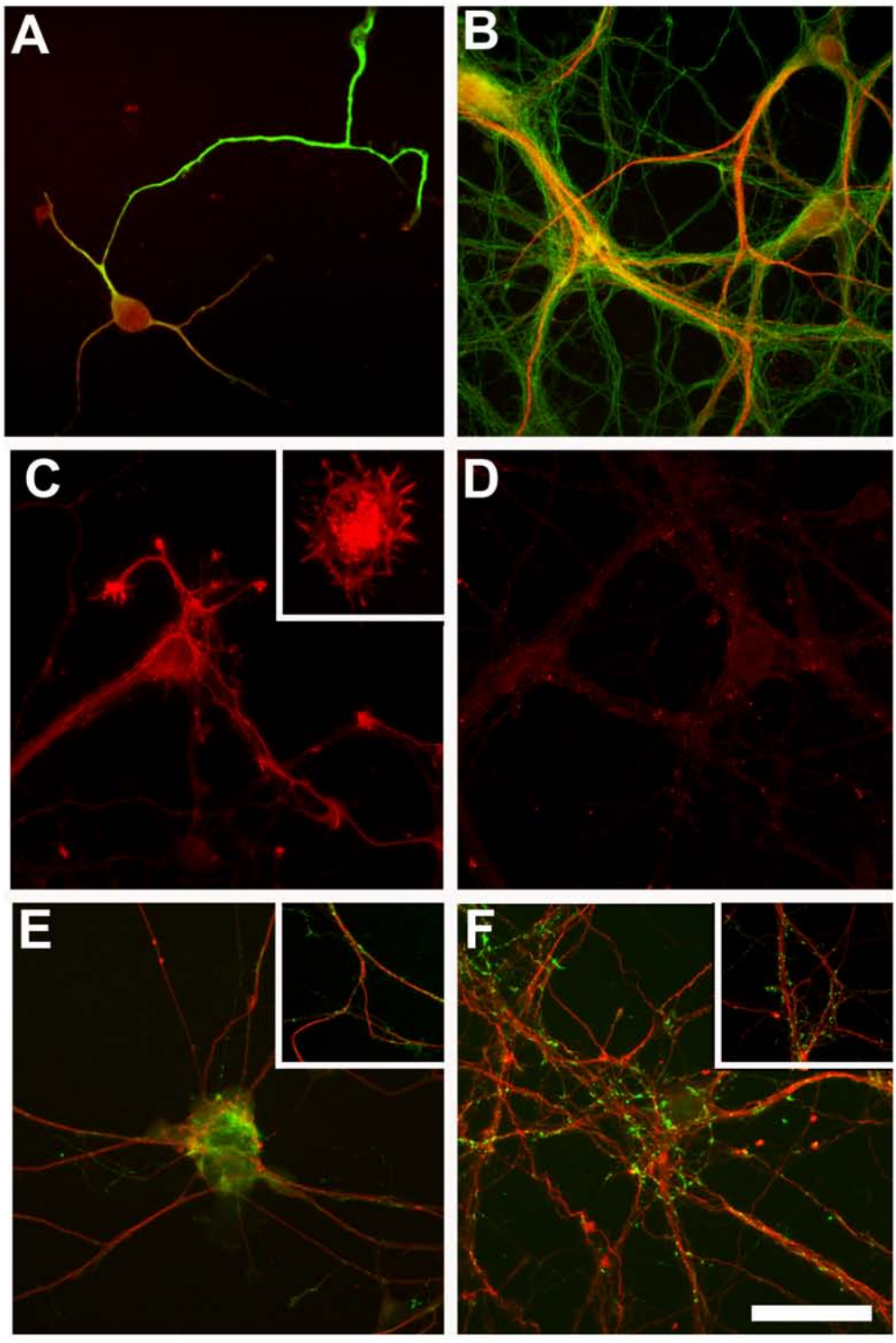

A
B
C
D
E
F

Figure 6.1 Immunocytochemistry of mouse cortical neurons *in vitro*.
Immmunocytochemistry was performed to investigate the maturation of neurons derived from wild type mice. (A) Immunocytochemistry at 7 DIV demonstrated neurons were polarised with MAP-2 positive dendrites (red) and a single tau immunopositive axon (green). (B) At 15 DIV tau (green) was abundant in axonal processes and MAP-2 (red) immunoreactivity was increased within dendrites. (C) GAP-43 (red) was enriched in cell bodies, axons, dendrites and growth cones (inset) at 7 DIV. (D) By 15 DIV there was minimal immunoreactivity for GAP-43 within the cortical neurons. (E) At 7 DIV colocalisation of βIII tubulin (red) and the synaptic vesicle marker synaptophysin (green) indicated that βIII tubulin was present throughout cell bodies and processes while synaptophysin (green) was confined to dendritic shafts (inset). (F) By 15 DIV synaptophysin was located in punctate positions lateral to the dendrite shaft (inset), indicating that mature synapses were present. Scale bar = 50μm, for inset 20μm

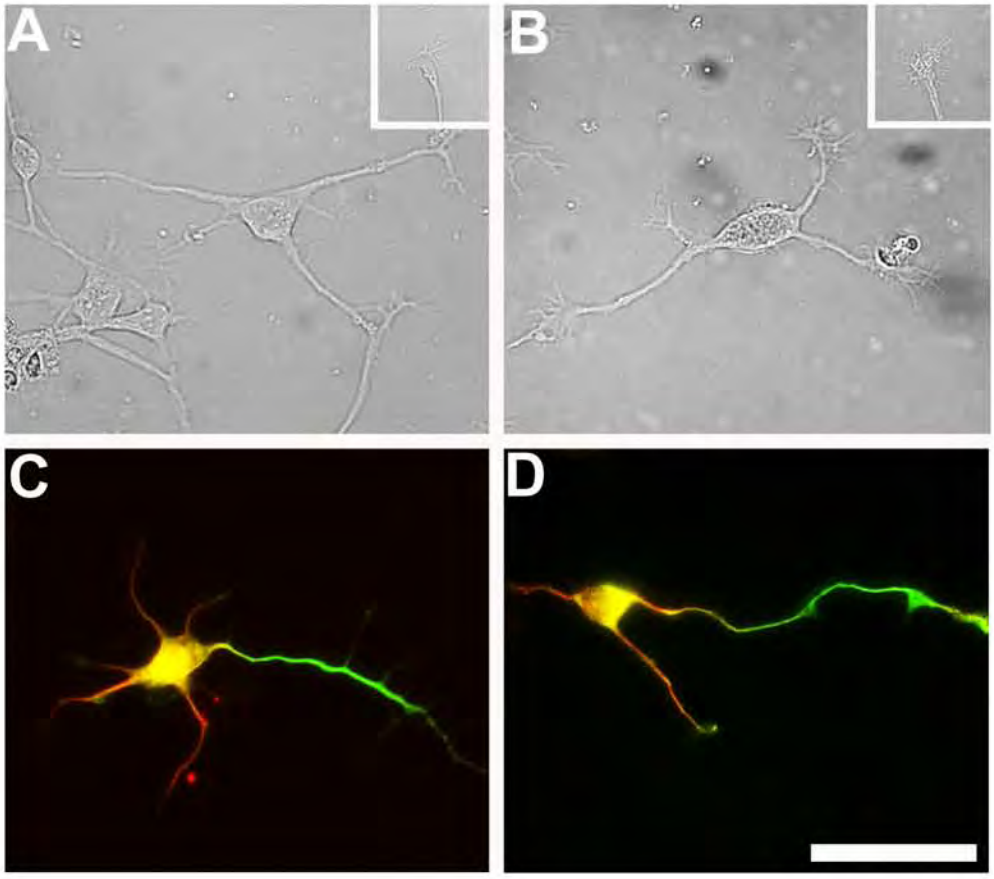

A
B
C
D

Figure 6.2 Immunocytochemistry of wildtype and NF-L-/- cortical neurons at 3 DIV. To visualise individual neuronal characteristics, both wildtype and NF-L-/- dissociated neurons were grown as a monolayer on a poly-L-lysin substrate. (A and B) DIC images demonstrated that (A) wildtype and (B) NF-L-/- cortical cultures adhered to the substrate and had extended neurites by 3 DIV, with no distinguishable differences between the two conditions. (A and B inset) These neurites were tipped with characteristic growth cones. (C and D) Immunolabelling for the microtubules associated proteins tau (green) and MAP-2 (red), demonstrated that both (C) wildtype and (D) NF-L-/- neurons had developed polarised neurites, with clearly defined dendrites (red) and a single axon (green) at 3 DIV. Scale bar = 50μm, for inset 20μm

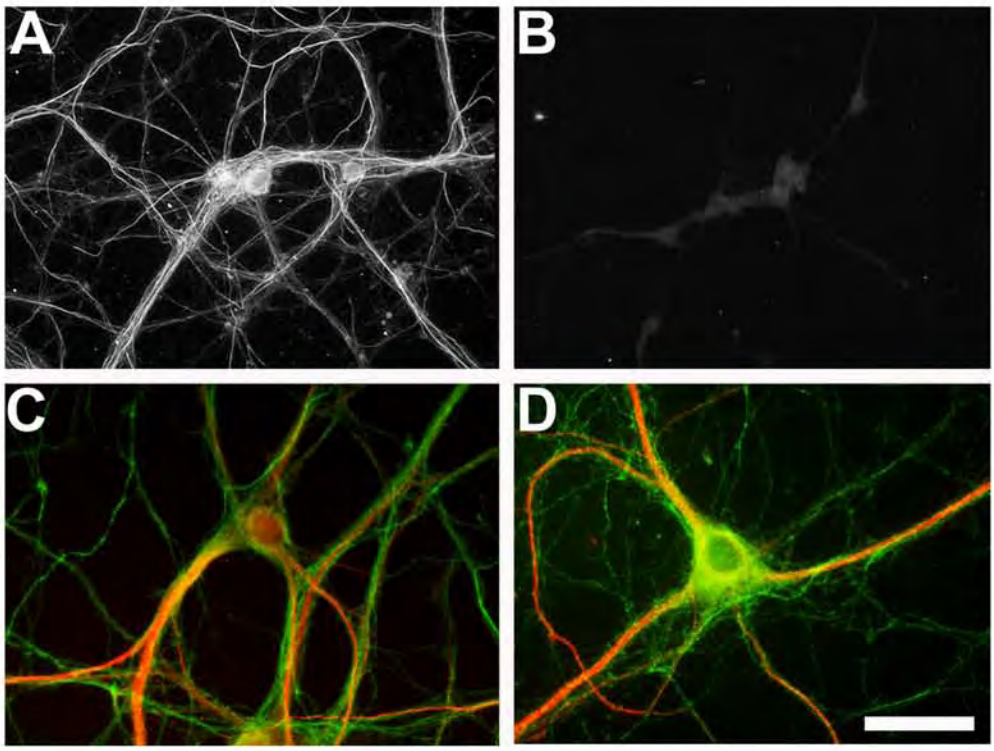

A
B
C
D

Figure 6.3 Wildtype and NF-L-/- neurons' expression of MAP-2 and Tau at 15 DIV. The immunoreactivity profile of 15 DIV NF-L-/- neurons was compared to wildtype controls. (A) Immunohistochemistry of the antibody direct at NF-L demonstrated that there was NF-L present in wildtype neurons throughout the cell bodies and processes. (B) There was no immunoreactivity for NF-L and the NF-L-/- neurons. (C and D) Immunolabelling for the microtubules associated proteins Tau (green) and MAP-2 (red), demonstrated that by 15 DIV in both (C) wildtype and (D) NF-L-/- neurons tau abundant in axonal processes and MAP-2 (red) immunoreactivity was present throughout all dendrites. Scale bar = 40μm

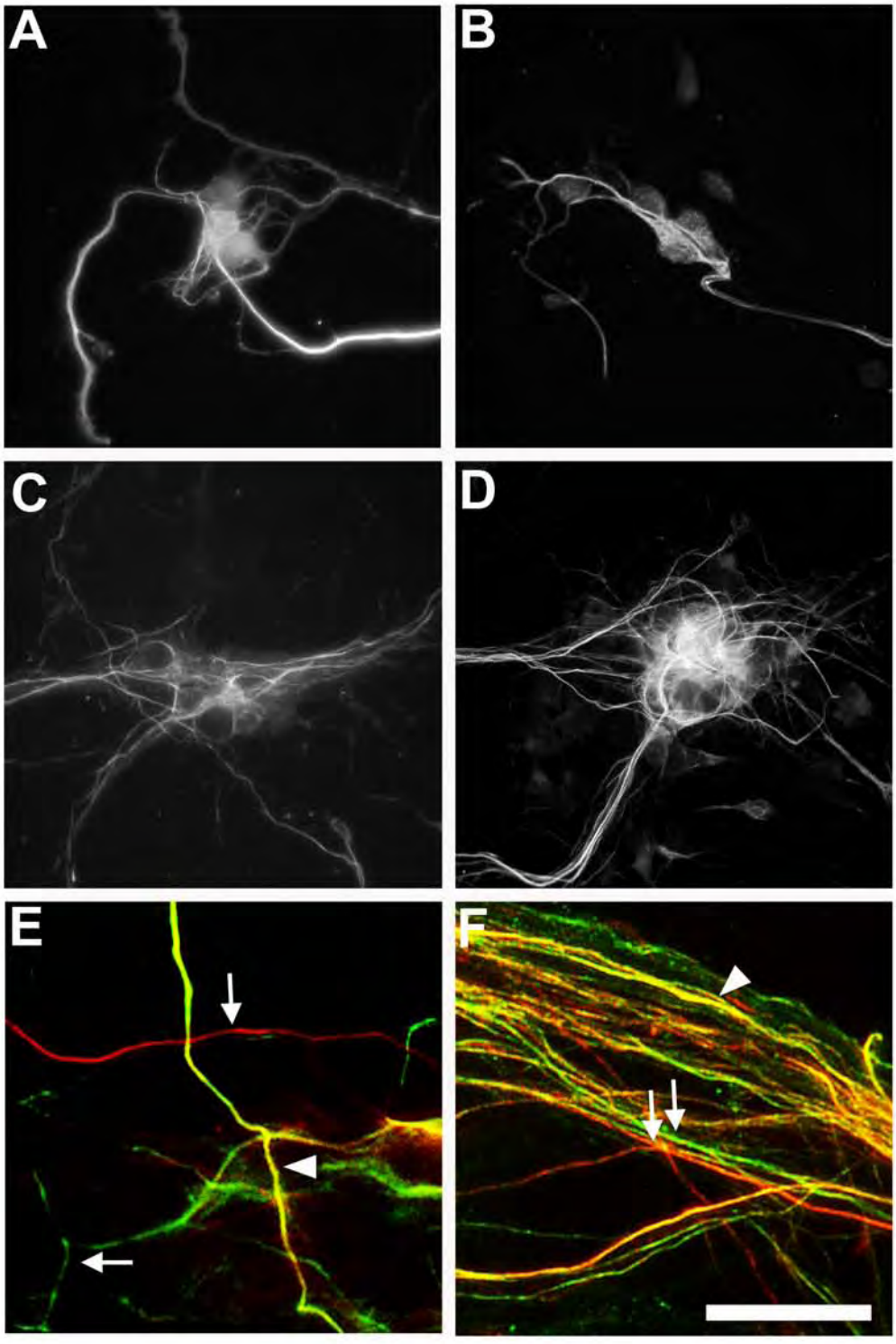

A
B
C
D
E
F

Figure 6.4 Immunolabelling of α-internexin and NF-M and 7 DIV in wildtype and NF-L-/- neurons. The immunoreactivity profile of developing NF-L-/- neurons was compared to wildtype controls. (A and B) Alpha internexin labelling at 7 DIV in (A) wildtype and (B) NF-L-/- neurons was predominately confined to neurite processes, with an increase in immunoreactivity for (B) NF-L-/- in comparison to (A) wildtype. (C and D) NF-M labelling at 7 DIV in (C) wildtype and (D) NF-L-/- neurons was also confined to neurite processes, however immunoreactivity was decreased for NF-L-/-. (E and F) Colocalisation investigations of α-internexin (red) and NF-M (green) in both (E) wildtype and (F) NF-L-/- neurons demonstrated that there were axons which were immunopositive for exclusively α-internexin (red) or NF-M (green) (arrows) as well as axons in which α-internexin and NF-M were co-localised (arrowheads). Scale bar = A-D, 50μm, E&F, 20μm

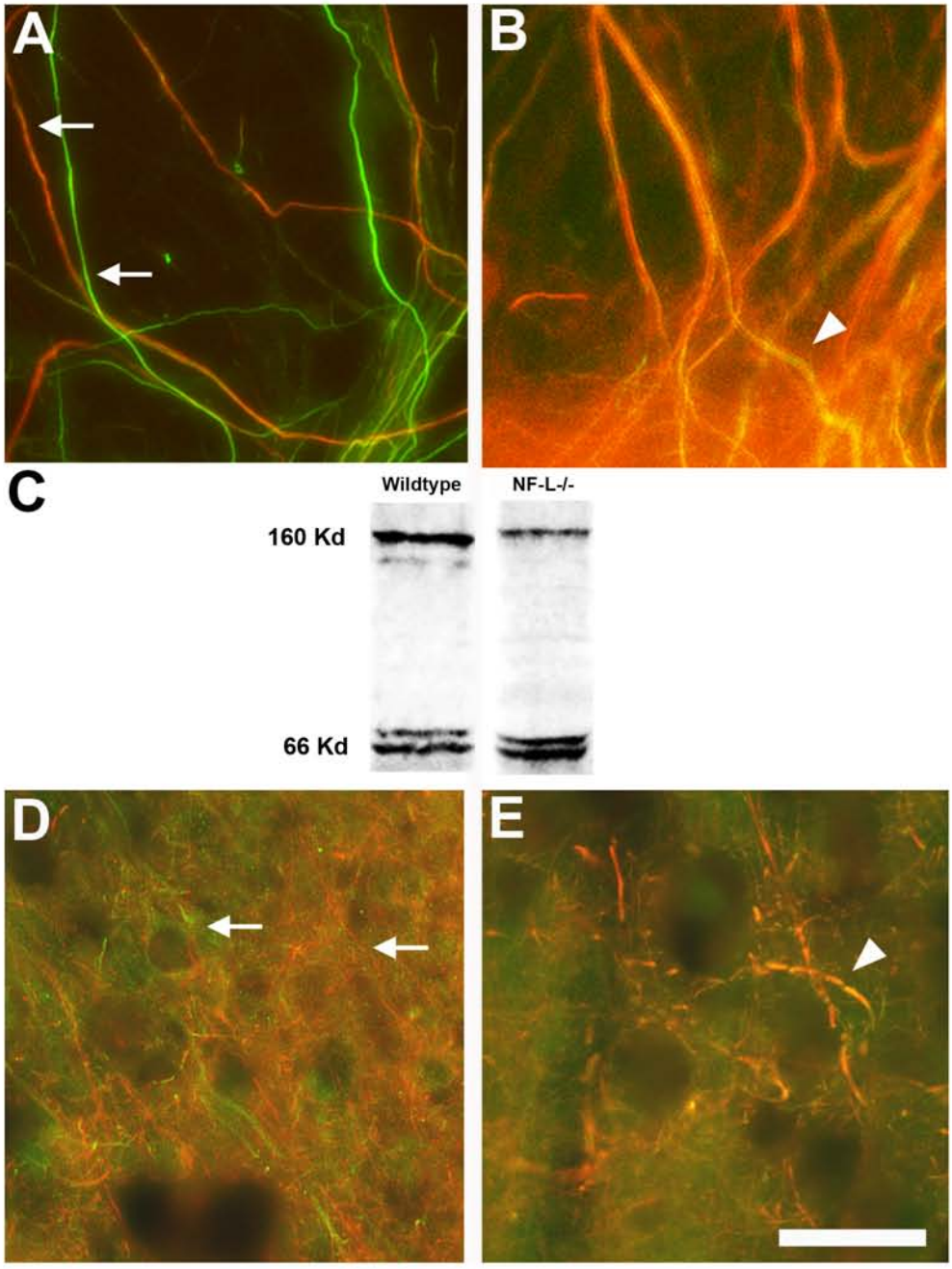
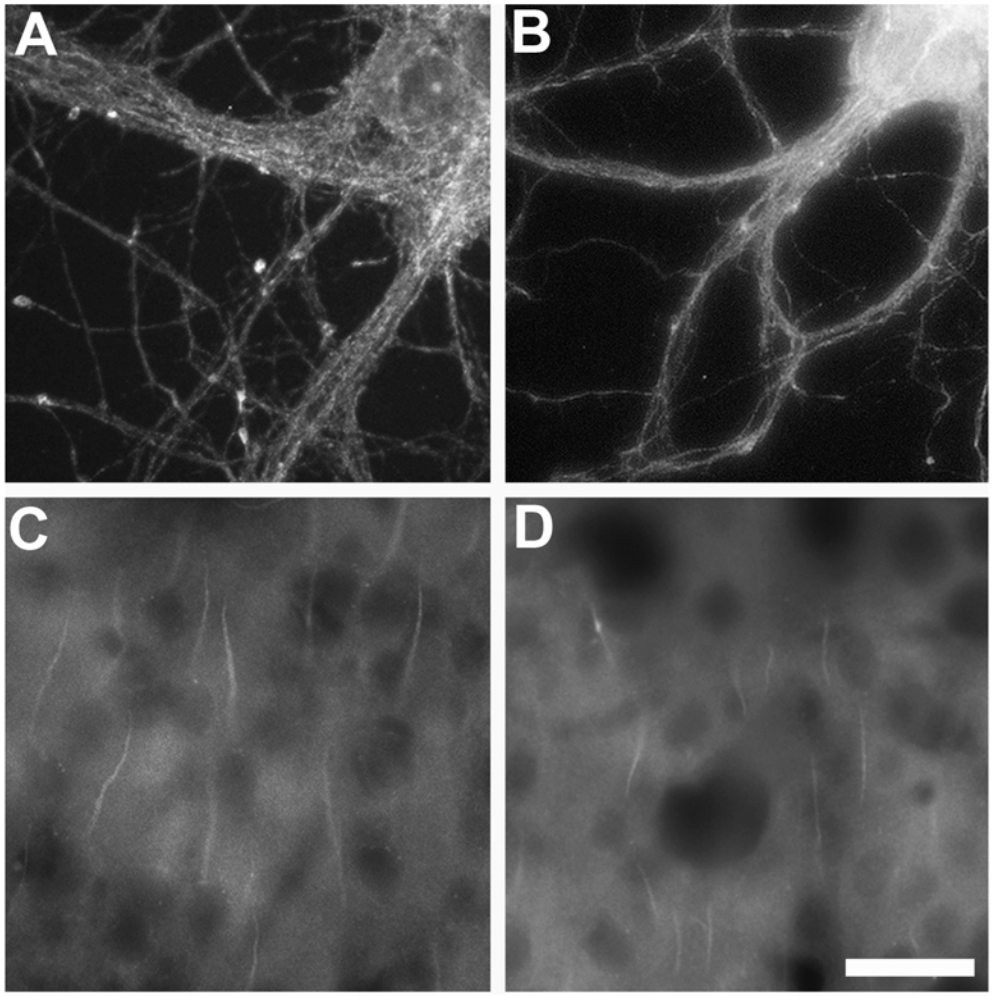

A
B
C
Wildtype
NF-L-/-
160 Kd
66 Kd
D
E

Figure 6.5 NF-M and α-internexin immunohistochemistry at 15 DIV in wildtype and NF-L-/- neurons. (A) Colocalisation investigations of α-internexin (red) and NF-M (green) in wildtype neurons at 15 DIV demonstrated that wildtype axons demonstrated a distinct expression of NF-M (green) or α-internexin (red) (arrows), with no colocalisation. (B) 15 DIV NF-L-/- axons demonstrate complete colocalisation of NF-M (green) and α-internexin (red)(arrowheads). (C) Immunoblot analysis of 15 DIV cortical neurons demonstrated that in comparison to wildtype, NF-L-/- neurons had a relatively decreased expression of NF-M (120 Kd) and increased expression of α-internexin (66kd). (D) Colocalisation immunohistochemistry within coronal brain sections, in the wildtype cortex also demonstrated that axons were immunopositive for exclusively α-internexin (red) or NF-M (green) within the neocortex (arrows). (E) NF-L-/- axons within the neocortex demonstrated a colocalisation of α-internexin (red) and NF-M (green) (arrowheads). Scale bar = 20μm


A
B
C
D

E

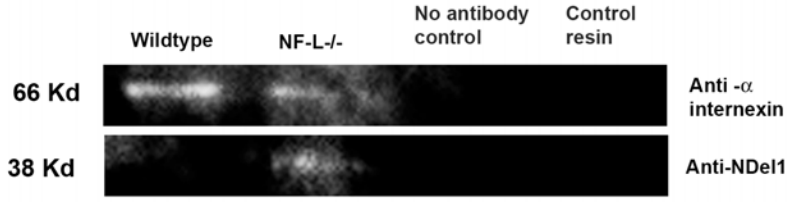

Wildtype
NF-L-/-
No antibody control
Control resin
66 Kd
38 Kd
Anti -α internexin
Anti-NDel1

Figure 6.6 Immunohistochemistry of interneuronal markers within the wildtype and NF-L-/- neocortex. (A) Labelling for MAP-2 in the wildtype neocortex demonstrated widespread immunoreactivity throughout the cortical layers, with a marginal decrease in immunoreactivity corresponding to layer IV. (B) There was also widespread immunoreactivity to MAP-2 within the NF-L-/- neocortex. (C and D) Immunolabelling for calretinin was throughout the cortical layers within both the (C) wildtype and (D) NF-L-/- neocortex, with an increase in density of calretinin positive cells corresponding to layer IV of the neocortex. Furthermore, there was very little immunoreactivity within layer I in both (C) wildtype and (D) NF-L-/- neocortex. (E and F) Parvalbumin expression was throughout the neocortex of (E) wildtype and (F) NF-L-/- mice, with a defined increase in immunoreactivity corresponding to layer V. Scale bar =100μm

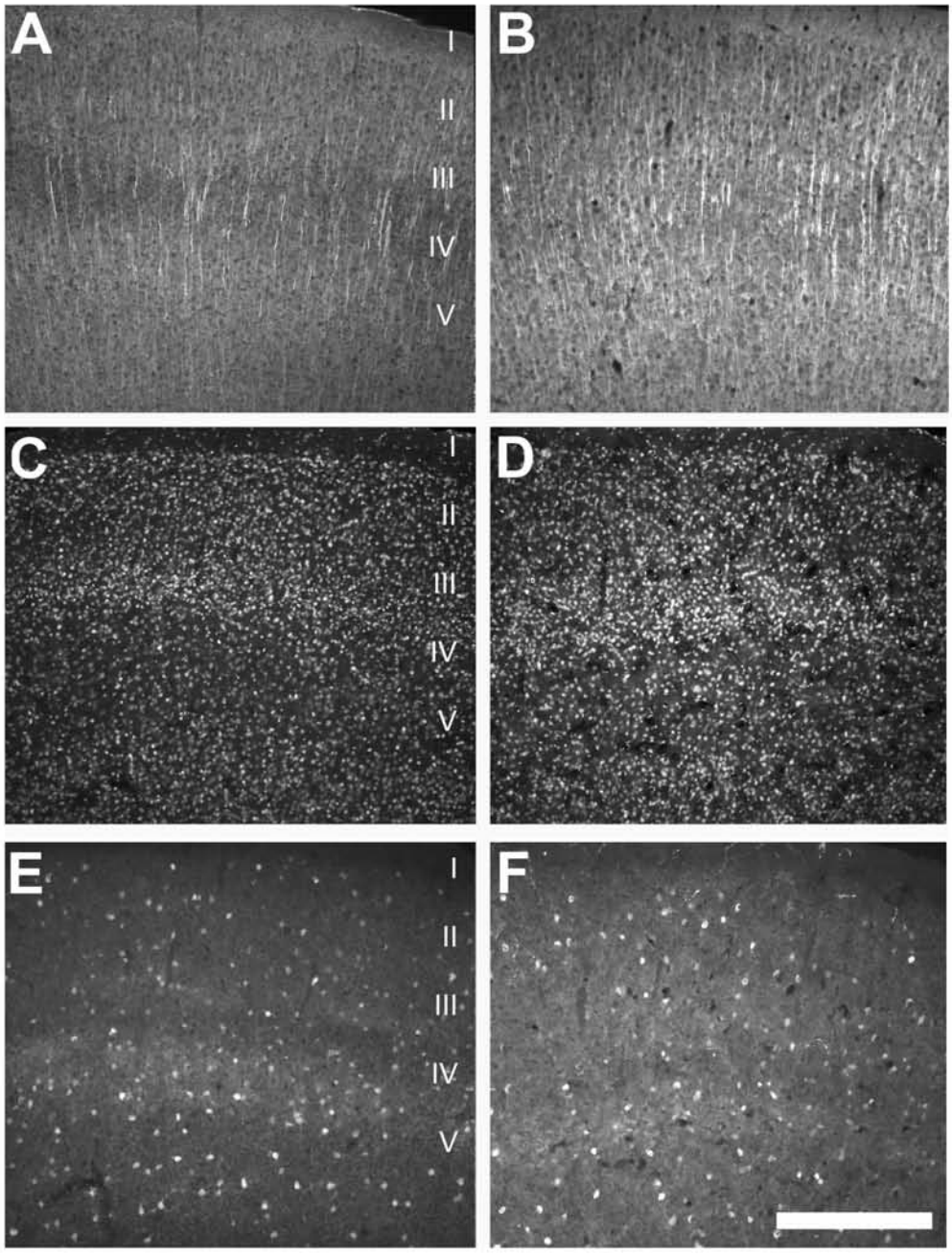

A
I
II
III
IV
V
B
C
I
II
III
IV
V
D
E
I
II
III
IV
V
F

Figure 6.7 Immunohistochemistry of neurofilament markers within the wildtype and NF-L-/- neocortex. (A) Immunohistochemistry on coronal sections demonstrated that SMI312 immunoreactivity was predominately confined to layer V and to a lesser extent layer III of the neocortex. (B) There was a reduced immunoreactivity for SMI312 within the NF-L-/- neocortex with no discernible cortical layers. (C and D) Additionally NF-M labelling was predominately confined to layers III and V within the (C) wildtype neocortex, (D) with no laminar expression present within the NF-L-/- neocortex. (E) Alpha internexin immunoreactivity was predominately confined to layers III and V of the wildtype neocortex. (F) Immunoreactivity for α-internexin was increased within the NF-L-/- neocortex compared to wildtype however the cortical layers were not distinguishable.
Scale bar =100μm

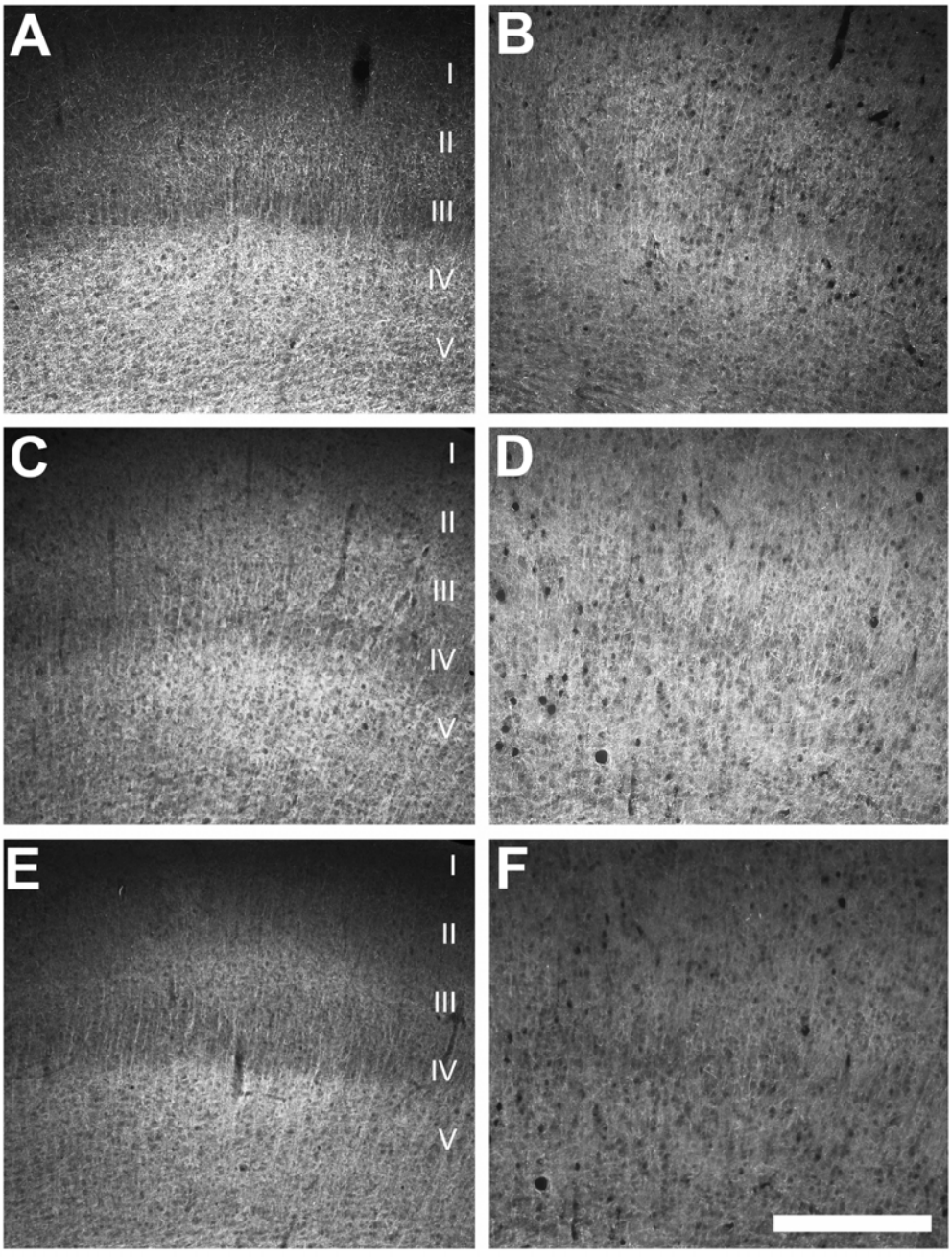

A
I
II
III
IV
V
B
C
I
II
III
IV
V
D
E
I
II
III
IV
V
F

Figure 6.8 NDel1 immunoreactivity in mature NF-L-/- neurons and coimmunoprecipitation with α-internexin. (A-D) Immunolabelling with an antibody direct at NDel1 demonstrated immunoreactivity both *in vitro* and *in vivo*, in mature NF-L-/- neurons. (A and B) NDel1 immunolabelling was present throughout cell bodies and axons at 15 DIV in both (A) wildtype and (B) NF-L-/- neurons. (C and D) *In vivo* brain sections demonstrated immunoreactivity within axon segments in both (C) wildtype and (D) NF-L-/- brains. (E) An immunoblot of immunoprecipitation with the polyclonal α-internexin antibody demonstrated that NDel1 coimmunoprecipitated with α-internexin exclusively in NF-L-/- neurons at 15 DIV. Both wildtype and NF-L-/- neurons precipitated α-internexin when probed with a polyclonal α-internexin antibody (column 1 and 2 or the first row respectively). However, only the NF-L-/- sample precipitated with α-internexin coimmunoprecipitated NDel1 on an immunoblot (column 2 row 2). Both control samples (no antibody control and a control resin, column 3 and 4 respectively) did no produce any detectable bands for either α-internexin or NDel1. Scale bar =50μm

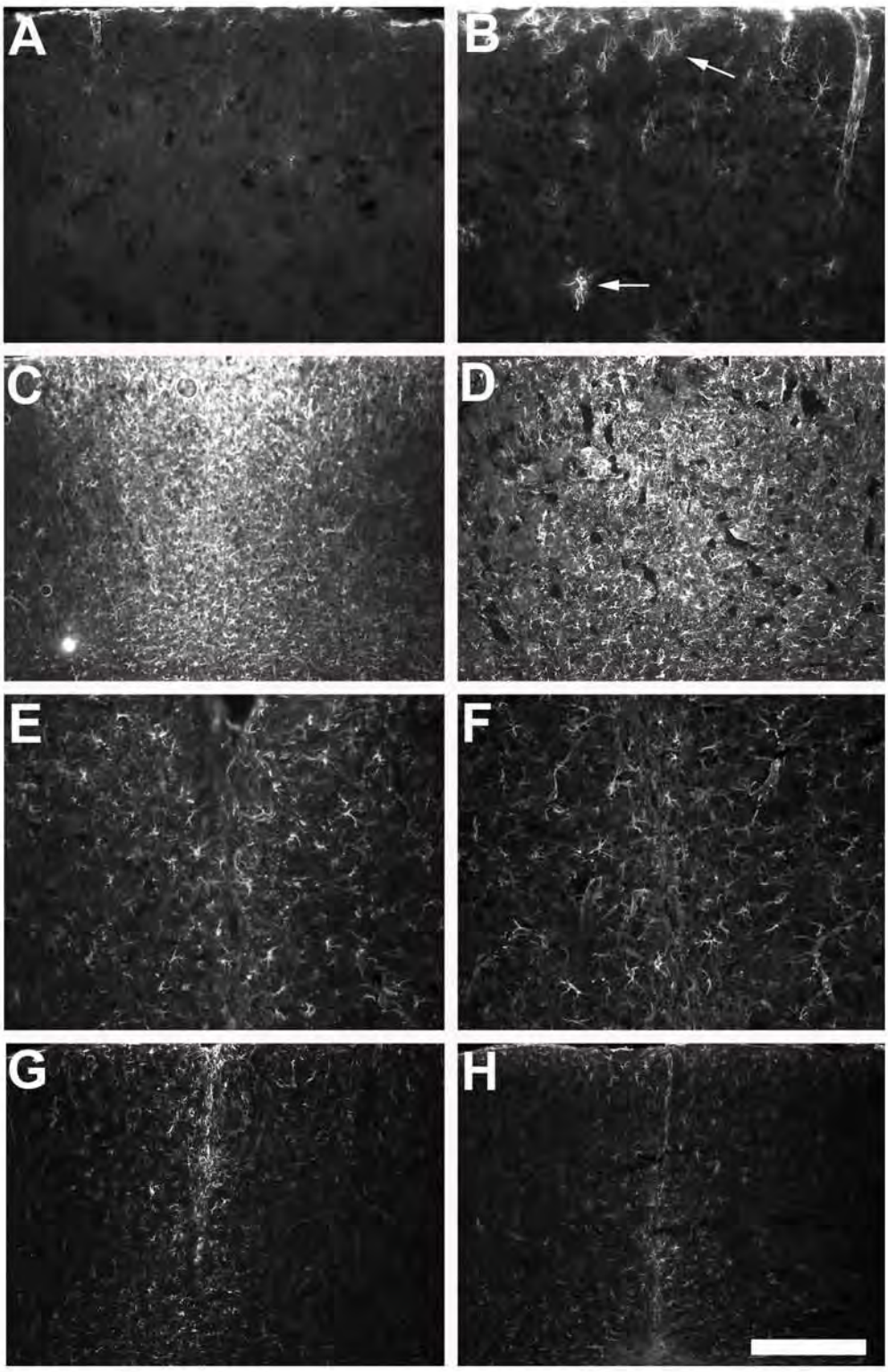

A
B
C
D
E
F
G
H

Figure 6.9 Time course of GFAP immunoreactivity following focal neocortical injury. (A and B) GFAP immunoreactivity within non-injured wildtype (A) and NF-L-/- (B) neocortex demonstrated an increase in GFAP immunopositive astrocytes within the neocortex of NF-L-/- mice (arrows). (C and D) At 4 DPI there was an increase in immunoreactivity for GFAP within the injury site in both (C) wildtype and (D) NF-L-/- neocortices. (E and F) By 7 DPI there was a localised increased expression of GFAP around the injury site within both (E) wildtype and (F) NF-L-/- neocortices. (G and H, wildtype and NF-L-/- respectively) Immunoreactivity was confined to a discreet central core by 14 DPI, demonstrating that an astrocyte infiltrated glial scar had formed. Scale bar =100μm

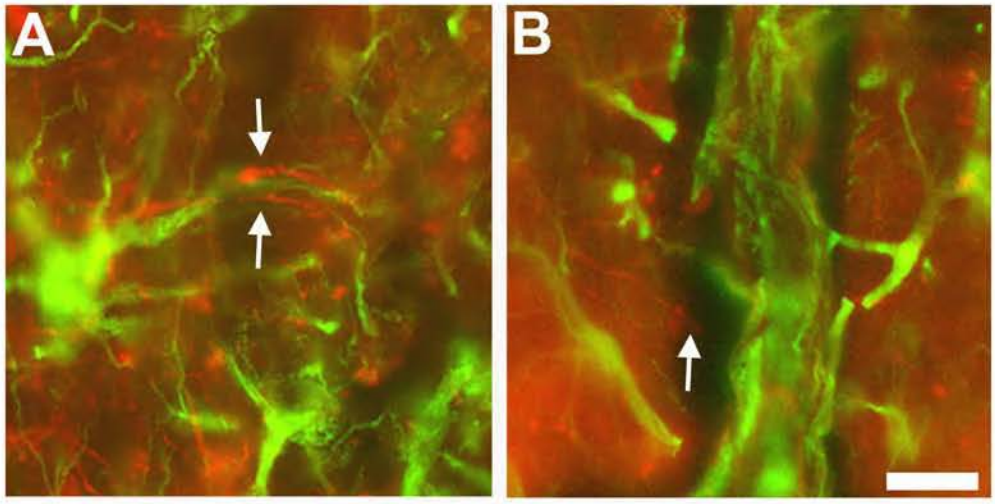

A
B

Figure 6.10 Immunofluorescent labeling a 4 DPI. (A and B) Coimmunohistochemistry for GFAP (green) and a combination of SMI312 and SMI32 (red) demonstrated that fine sprout like structures were transversing the injury site at 4 DPI (arrows) in both the (A) wildtype and (B) NF-L-/- injury site. Scale bar =30μm

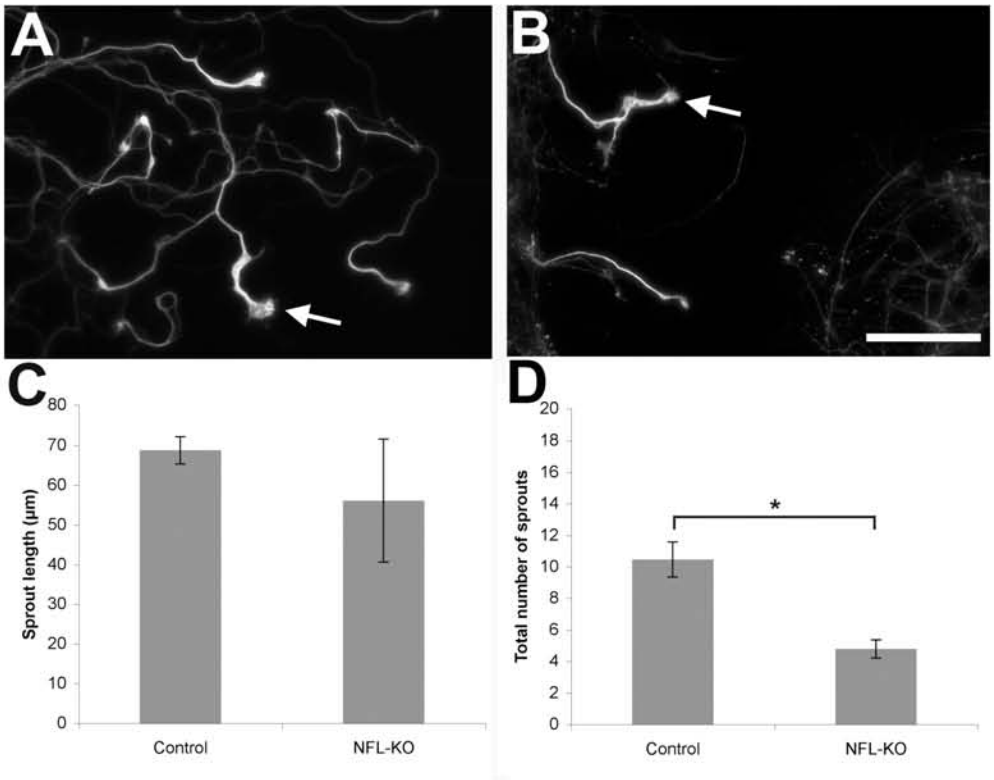

A
B
C
Sprout length (µm)
80
70
60
50
40
30
20
10
0
Control
NFL-KO
D
Total number of sprouts
20
18
16
14
12
10
8
6
4
2
0
*
Control
NFL-KO

Figure 6.11 Quantitation of the number and mean length of NF-L-/- sprouts within the injury site *in vitro*. The sprouting response was investigated at 24 hrs post injury in relatively mature (15 DIV) neurons *in vitro.* (A) Tau labelling of wildtype neurons at 24 hrs PI demonstrated that fine sprouts were transversing the injury site, tipped with characteristic growth cones (arrows). (B) Tau positive regenerative sprouts, tipped with growth cones (arrows), were also present within the injury site of NF-L-/- cortical neurons. (C) There was no significant difference between the mean sprout lengths for NF-L-/- regenerating sprouts in comparison to wildtype control. (D) However there was a significant reduction in the mean number of spouts, within each injury site, in NF-L-/ neurons in comparison to wildtype control. Scale bar =20µm